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Christen M. Anderson

Title

PRODUCTION OF ADENINE NUCLEOTIDE
TRANSLOCATOR (ANT), NOVEL ANT LIGANDS AND
SCREENING ASSAYS THEREFOR

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APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents

ADDRESS TO:

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(Submit an original and a duplicate for fee processing)2. ☒ Specification [Total Pages] 109
(preferred arrangement set forth below)

- Descriptive Title of the Invention
- Cross References to Related Applications
- Statement Regarding Fed sponsored R & D
- Reference to Microfiche Appendix
- Background of the Invention
- Brief Summary of the Invention
- Brief Description of the Drawings (if filed)
- Detailed Description
- Claim(s)
- Abstract of the Disclosure

3. ☒ Drawing(s) (35 USC 113) [Total Sheets] 10

4. Oath or Declaration [Total Pages]

- a. ☐ Newly executed (original or copy)
- b. ☐ Copy from a prior application (37 CFR 1.63(d))
(for continuation/divisional with Box 17 completed)
 - i. ☐ DELETION OF INVENTOR(S)
Signed statement attached deleting
inventor(s) named in the prior application,
see 37 CFR 1.63(d)(2) and 1.33(b)

5. ☐ Incorporation By Reference (useable if box 4b is checked)
The entire disclosure of the prior application, from which a
copy of the oath or declaration is supplied under Box 4b, is
considered to be part of the disclosure of the accompanying
application and is hereby incorporated by reference therein.6. ☐ Microfiche Computer Program (Appendix)7. Nucleotide and Amino Acid Sequence Submission
(if applicable, all necessary)

- a. ☐ Computer-Readable Copy
- b. ☐ Paper Copy (identical to computer copy)
- c. ☐ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment Papers (cover sheet & document(s))9. ☐ 37 CFR 3.73(b) Statement (when there is an assignee) ☐ Power of Attorney10. ☐ English Translation Document (if applicable)11. ☐ Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations12. ☐ Preliminary Amendment13. ☒ Return Receipt Postcard14. ☐ Small Entity Statement(s) ☐ Statement filed in prior application,
Status still proper and desired15. ☐ Certified Copy of Priority Document(s)
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Prior application information Examiner _____ Group / Art Unit _____

☐ Claims the benefit of Provisional Application No. _____

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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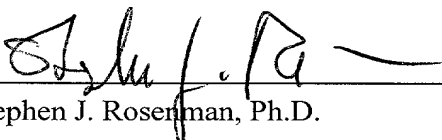
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Enclosures:

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Specification, Claims, Abstract (109 pages)

10 Sheets of Drawings (Figures 1-10)

PRODUCTION OF ADENINE NUCLEOTIDE TRANSLOCATOR (ANT),
NOVEL ANT LIGANDS AND SCREENING ASSAYS THEREFOR

FIELD OF THE INVENTION

The invention relates to the adenine nucleotide translocator (ANT) protein that is found in mitochondria of eukaryotic cells. More particularly, the invention relates to the production of ANT polypeptides and ANT fusion proteins using recombinant DNA technology; to novel labeled ligands for ANT proteins; and to assays useful for identifying and isolating ANT proteins and for screening compounds that interact with ANT, including high throughput screening.

10 BACKGROUND OF THE INVENTION

Mitochondria are the main energy source in cells of higher organisms, and these organelles provide direct and indirect biochemical regulation of a wide array of cellular respiratory, oxidative and metabolic processes. These include electron transport chain (ETC) activity, which drives oxidative phosphorylation to produce metabolic energy in the form of adenosine triphosphate (ATP), and which also underlies a central mitochondrial role in intracellular calcium homeostasis.

Mitochondrial ultrastructural characterization reveals the presence of an outer mitochondrial membrane that serves as an interface between the organelle and the cytosol, a highly folded inner mitochondrial membrane that appears to form attachments to the outer membrane at multiple sites, and an intermembrane space between the two mitochondrial membranes. The subcompartment within the inner mitochondrial membrane is commonly referred to as the mitochondrial matrix. (For a review, *see, e.g.,* Ernster et al., 1981 *J. Cell Biol.* 91:227s.) The cristae, originally postulated to occur as infoldings of the inner mitochondrial membrane, have recently been characterized using three-dimensional electron tomography as also including tube-like conduits that may form networks, and that can be connected to the inner membrane by open, circular (30 nm diameter) junctions (Perkins et al., 1997, *Journal of Structural Biology* 119:260). While the outer membrane is freely permeable to ionic and non-ionic

solutes having molecular weights less than about ten kilodaltons, the inner mitochondrial membrane exhibits selective and regulated permeability for many small molecules, including certain cations, and is impermeable to large ($> \sim 10$ kDa) molecules.

5 Altered or defective mitochondrial activity, including but not limited to failure at any step of the ETC, may result in catastrophic mitochondrial collapse that has been termed “permeability transition” (PT) or “mitochondrial permeability transition” (MPT). According to generally accepted theories of mitochondrial function, proper ETC respiratory activity requires maintenance of an electrochemical potential ($\Delta\Psi_m$) in
10 the inner mitochondrial membrane by a coupled chemiosmotic mechanism. Altered or defective mitochondrial activity may dissipate this membrane potential, thereby preventing ATP biosynthesis and halting the production of a vital biochemical energy source. In addition, mitochondrial proteins such as cytochrome c may leak out of the mitochondria after permeability transition and may induce the genetically programmed
15 cell suicide sequence known as apoptosis or programmed cell death (PCD).

MPT may result from direct or indirect effects of mitochondrial genes, gene products or related downstream mediator molecules and/or extramitochondrial genes, gene products or related downstream mediators, or from other known or unknown causes. Loss of mitochondrial potential therefore may be a critical event in
20 the progression of diseases associated with altered mitochondrial function, including degenerative diseases.

Mitochondrial defects, which may include defects related to the discrete mitochondrial genome that resides in mitochondrial DNA and/or to the extramitochondrial genome, which includes nuclear chromosomal DNA and other
25 extramitochondrial DNA, may contribute significantly to the pathogenesis of diseases associated with altered mitochondrial function. For example, alterations in the structural and/or functional properties of mitochondrial components comprised of subunits encoded directly or indirectly by mitochondrial and/or extramitochondrial DNA, including alterations deriving from genetic and/or environmental factors or
30 alterations derived from cellular compensatory mechanisms, may play a role in the

pathogenesis of any disease associated with altered mitochondrial function. A number of degenerative diseases are thought to be caused by, or to be associated with, alterations in mitochondrial function. These include Alzheimer's Disease (AD); diabetes mellitus; Parkinson's Disease; Huntington's disease; dystonia; Leber's hereditary optic neuropathy; schizophrenia; mitochondrial encephalopathy, lactic acidosis, and stroke (MELAS); cancer; psoriasis; hyperproliferative disorders; mitochondrial diabetes and deafness (MIDD) and myoclonic epilepsy ragged red fiber syndrome. The extensive list of additional diseases associated with altered mitochondrial function continues to expand as aberrant mitochondrial or mitonuclear activities are implicated in particular disease processes.

A hallmark pathology of AD and potentially other diseases associated with altered mitochondrial function is the death of selected cellular populations in particular affected tissues, which results from apoptosis (also referred to as "programmed cell death" or PCD) according to a growing body of evidence. Mitochondrial dysfunction is thought to be critical in the cascade of events leading to apoptosis in various cell types (Kroemer et al., *FASEB J.* 9:1277-87, 1995), and may be a cause of apoptotic cell death in neurons of the AD brain. Altered mitochondrial physiology may be among the earliest events in PCD (Zamzami et al., *J. Exp. Med.* 182:367-77, 1995; Zamzami et al., *J. Exp. Med.* 181:1661-72, 1995) and elevated reactive oxygen species (ROS) levels that result from such altered mitochondrial function may initiate the apoptotic cascade (Ausserer et al., *Mol. Cell. Biol.* 14:5032-42, 1994).

Thus, in addition to their role in energy production in growing cells, mitochondria (or, at least, mitochondrial components) participate in apoptosis (Newmeyer et al., 1994, *Cell* 79:353-364; Liu et al., 1996, *Cell* 86:147-157). Apoptosis is apparently also required for, *inter alia*, normal development of the nervous system and proper functioning of the immune system. Moreover, some disease states are thought to be associated with either insufficient (e.g., cancer, autoimmune diseases) or excessive (e.g., stroke damage, AD-associated neurodegeneration) levels of apoptosis. For general reviews of apoptosis, and the role of mitochondria therein, see Green and

Reed (1998, *Science* 281:1309-1312), Green (1998, *Cell* 94:695-698) and Kromer (1997, *Nature Medicine* 3:614-620). Hence, agents that effect apoptotic events, including those associated with mitochondrial components, might have a variety of palliative, prophylactic and therapeutic uses.

5 The adenine nucleotide translocator (ANT), a nuclear encoded polypeptide that is a major component of the inner mitochondrial membrane, is responsible for mediating transport of ADP and ATP across the mitochondrial inner membrane. For example, ANT is believed to mediate stoichiometric ATP/proton exchange across the inner mitochondrial membrane, and ANT inhibitors (such as
10 atractyloside or bongkreikic acid) induce MPT under certain conditions. Three human ANT isoforms have been described that differ in their tissue expression patterns and other mammalian ANT homologues have been described. (See, *e.g.*, Wallace et al., 1998 in *Mitochondria & Free Radicals in Neurodegenerative Diseases*, Beal, Howell and Bodis-Wollner, Eds., Wiley-Liss, New York, pp. 283-307, and references cited
15 therein.) ANT has also been implicated as an important molecular component of the mitochondrial permeability transition pore, a Ca^{2+} -regulated inner membrane channel that, as described above, plays an important modulating role in apoptotic processes.

As inner mitochondrial membrane proteins are believed to possess multiple hydrophobic membrane spanning domains, ANT polypeptides may exhibit,
20 *inter alia*, poor intracellular solubility, toxic accumulations and/or the formation of inclusion bodies and other deleterious effects on respiratory homeostasis within a host cell due to ANT biological activity. Consequently, those having ordinary skill in the art have heretofore been unable to produce ANT reliably or in sufficient quantities for a variety of uses, such as those provided herein. Because of the significance of
25 mitochondria to respiratory, metabolic and apoptotic processes, and in view of the prominent role played by ANT in these and other mitochondrial activities, there is clearly a need for compositions and methods that permit the production of ANT proteins, including ANT fusion proteins; for novel ANT ligands; for methods to identify and isolate ANT proteins; and for methods of identifying and isolating agents that
30 interact with ANT.

The present invention fulfills these needs and provides other related advantages. These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures or compositions (*e.g.*, plasmids, vectors, etc.), and are therefore incorporated by reference in their entireties.

SUMMARY OF THE INVENTION

In its various aspects and embodiments the invention is directed to:

A recombinant expression construct comprising at least one regulated promoter operably linked to a first nucleic acid encoding an adenine nucleotide translocator polypeptide; further comprising at least one additional nucleic acid sequence that regulates transcription; wherein the additional nucleic acid sequence that regulates transcription encodes a repressor of said regulated promoter. 1 wherein the adenine nucleotide translocator polypeptide comprises a human adenine nucleotide translocator polypeptide. 15 wherein the human adenine nucleotide translocator polypeptide is ANT1; wherein the human adenine nucleotide translocator polypeptide is ANT2.

wherein the human adenine nucleotide translocator polypeptide is ANT3; wherein the adenine nucleotide translocator polypeptide is expressed as a fusion protein with a polypeptide product of a second nucleic acid sequence; wherein the polypeptide product of said second nucleic acid sequence is an enzyme; wherein said fusion protein localizes to membranes; wherein said membranes are mitochondrial membranes; wherein the adenine nucleotide translocator polypeptide is expressed as a fusion protein with at least one product of a second nucleic acid sequence encoding a polypeptide 25 cleavable by a protease, said adenine nucleotide translocator polypeptide being separable from the fusion protein by cleavage with the protease; A host cell comprising a recombinant expression construct as provided; wherein the host cell is a prokaryotic cell; wherein the host cell is a eukaryotic cell; wherein the eukaryotic cell is selected from the group consisting of a yeast cell, an insect cell and a mammalian cell; wherein

the insect cell is an Sf9 cell; at lacks at least one isoform of an endogenous adenine nucleotide translocator; in which expression of at least one gene encoding an endogenous adenine nucleotide translocator isoform is substantially impaired.

A recombinant expression construct comprising at least one promoter
 5 operably linked to a nucleic acid molecule comprising a first nucleic acid sequence and a second nucleic acid sequence, said first nucleic acid sequence encoding an animal adenine nucleotide translocator polypeptide wherein the adenine nucleotide translocator polypeptide is expressed as a fusion protein with a polypeptide product of said second nucleic acid sequence; wherein the polypeptide product of said second nucleic acid
 10 sequence is an enzyme; wherein said fusion protein localizes to membranes; wherein said membranes are mitochondrial membranes; further comprising at least one additional nucleic acid sequence that regulates transcription; wherein the additional nucleic acid sequence that regulates transcription encodes a repressor of said promoter; wherein the adenine nucleotide translocator polypeptide comprises a human adenine
 15 nucleotide translocator polypeptide; wherein the human adenine nucleotide translocator polypeptide is ANT1; wherein the human adenine nucleotide translocator polypeptide is ANT2; wherein the human adenine nucleotide translocator polypeptide is ANT3; wherein the adenine nucleotide translocator polypeptide is expressed as a fusion protein with at least one product of a second nucleic acid sequence encoding a polypeptide
 20 cleavable by a protease, said adenine nucleotide translocator polypeptide being separable from the fusion protein by cleavage with the protease; a host cell comprising a recombinant expression construct as just described; wherein the host cell is a prokaryotic cell; wherein the host cell is a eukaryotic cell; wherein the eukaryotic cell is selected from the group consisting of a yeast cell, an insect cell and a mammalian cell;
 25 wherein the insect cell is an Sf9 cell; that lacks at least one isoform of an endogenous adenine nucleotide translocator; in which expression of at least one gene encoding an endogenous adenine nucleotide translocator isoform is substantially impaired; wherein the expression construct is a recombinant viral expression construct;

A method of producing a recombinant adenine nucleotide translocator
 30 polypeptide, comprising; culturing a host cell comprising a recombinant expression

construct comprising at least one regulated promoter operably linked to a first nucleic acid encoding an adenine nucleotide translocator polypeptide;

A method of producing a recombinant adenine nucleotide translocator polypeptide, comprising culturing a host cell comprising a recombinant expression construct comprising at least one promoter operably linked to a nucleic acid molecule comprising a first nucleic acid sequence and a second nucleic acid sequence, said first nucleic acid sequence encoding an animal adenine nucleotide translocator polypeptide wherein the adenine nucleotide translocator polypeptide is expressed as a fusion protein with a polypeptide product of said second nucleic acid sequence;

A method of producing a recombinant adenine nucleotide translocator polypeptide, comprising culturing a host cell infected with the recombinant viral expression construct as provided above.

An ANT polypeptide produced by the methods just described.

An isolated human adenine nucleotide translocator polypeptide; wherein the human adenine nucleotide translocator polypeptide is recombinant ANT1 or a variant or fragment thereof; wherein the human adenine nucleotide translocator polypeptide is recombinant ANT2 or a variant or fragment thereof; wherein the human adenine nucleotide translocator polypeptide is recombinant ANT3 or a variant or fragment thereof;

An isolated human adenine nucleotide translocator fusion protein comprising an adenine translocator polypeptide fused to at least one additional polypeptide sequence; wherein said one additional polypeptide sequence is an enzyme sequence or a variant or fragment thereof; wherein said fusion protein localizes to membranes; wherein said membranes are mitochondrial membranes;

An isolated human adenine nucleotide translocator fusion protein comprising an adenine translocator polypeptide fused to at least one additional polypeptide sequence cleavable by a protease, said adenine nucleotide translocator polypeptide being separable from the fusion protein by cleavage with the protease.

An isolated adenine nucleotide translocator fusion protein comprising a first polypeptide that is an animal adenine translocator polypeptide fused to at least one

additional polypeptide sequence; wherein said one additional polypeptide sequence is an enzyme sequence or a variant or fragment thereof; that localizes to membranes; wherein said membranes are mitochondrial membranes.

5 An isolated recombinant animal adenine nucleotide translocator fusion protein comprising an adenine translocator polypeptide fused to at least one additional polypeptide sequence cleavable by a protease, said adenine nucleotide translocator polypeptide being separable from the fusion protein by cleavage with the protease; wherein the additional polypeptide sequence is a polypeptide having affinity for a ligand.

10 A method for determining the presence of an ANT polypeptide in a biological sample comprising contacting a biological sample suspected of containing an ANT polypeptide with an ANT ligand under conditions and for a time sufficient to allow binding of the ANT ligand to an ANT polypeptide; and detecting the binding of the ANT ligand to an ANT polypeptide, and therefrom determining the presence of an
 15 ANT polypeptide in said biological sample; wherein the adenine nucleotide translocator polypeptide comprises a human adenine nucleotide translocator polypeptide; wherein the human adenine nucleotide translocator polypeptide is ANT1; wherein the human adenine nucleotide translocator polypeptide is ANT2; wherein the human adenine nucleotide translocator polypeptide is ANT3; wherein the ANT ligand comprises
 20 atractyloside substituted at 6' hydroxyl to form an atractyloside derivative; wherein the atractyloside is detectably substituted at the 6' hydroxyl to form a detectable atractyloside derivative; wherein the detectable atractyloside derivative comprises a radiolabeled substituent; wherein the radiolabeled substituent is selected from the group consisting of ^{125}I , ^{131}I , ^3H , ^{14}C and ^{35}S ; wherein the detectable atractyloside
 25 derivative comprises a fluorescent substituent; wherein the ANT ligand further comprises a Eu^{2+} atom complexed to the atractyloside derivative; wherein the detectable atractyloside derivative comprises covalently bound biotin; wherein the atractyloside molecule is substituted at 6' hydroxyl with an amine or an amine containing functionality to form an amine modified atractyloside derivative; wherein the

atractyloside molecule is a carboxyatractyloside molecule that is substituted at 6' hydroxyl to form an atractyloside derivative that is a carboxyatractyloside derivative.

A method for isolating ANT from a biological sample, comprising contacting a biological sample suspected of containing an ANT polypeptide with an ANT ligand under conditions and for a time sufficient to allow binding of the ANT ligand to an ANT polypeptide; and recovering the ANT polypeptide, and thereby isolating ANT from a biological sample; wherein the ANT ligand is covalently bound to a solid phase; wherein the ANT ligand is non-covalently bound to a solid phase.

A method for identifying an agent that binds to an ANT polypeptide, comprising contacting a candidate agent with a host cell expressing at least one recombinant ANT polypeptide under conditions and for a time sufficient to permit binding of the agent to said recombinant ANT polypeptide; and detecting binding of said agent to the recombinant ANT; wherein the host cell is a prokaryotic cell; wherein the prokaryotic cell is an *E. coli* cell; wherein the host cell is a eukaryotic cell; wherein the eukaryotic cell is selected from the group consisting of a yeast cell, an insect cell and a mammalian cell; wherein the insect cell is an Sf9 cell; wherein the host cell lacks at least one isoform of an endogenous adenine nucleotide translocator; wherein host cell expression of at least one gene encoding an endogenous adenine nucleotide translocator isoform is substantially impaired.

A method for identifying an agent that binds to an ANT polypeptide, comprising contacting a candidate agent with a biological sample containing at least one recombinant ANT polypeptide under conditions and for a time sufficient to permit binding of the agent to said ANT polypeptide; and detecting binding of said agent to the recombinant ANT polypeptide.

A method for identifying an agent that interacts with an ANT polypeptide comprising contacting a biological sample containing recombinant ANT with a detectable ANT ligand in the presence of a candidate agent; and comparing binding of the detectable ANT ligand to recombinant ANT in the absence of said agent to binding of the detectable ANT ligand to recombinant ANT in the presence of said agent, and therefrom identifying an agent that interacts with an ANT polypeptide.

An ANT ligand comprising atractyloside substituted at the 6' hydroxyl to form an atractyloside derivative; wherein the atractyloside is detectably substituted at the 6' hydroxyl to form a detectable atractyloside derivative; wherein the detectable atractyloside derivative comprises a radiolabeled substituent; wherein the radiolabeled substituent is selected from the group consisting of ^{125}I , ^{131}I , ^3H , ^{14}C and ^{35}S ; wherein the detectable atractyloside derivative comprises a fluorescent substituent; further comprising a Eu^{2+} atom complexed to the atractyloside derivative; wherein the detectable atractyloside derivative comprises covalently bound biotin; wherein the atractyloside molecule is substituted at 6' hydroxyl with an amine or an amine containing functionality to form an amine modified atractyloside derivative; wherein the atractyloside molecule is a carboxyatractyloside molecule that is substituted at 6' hydroxyl to form an atractyloside derivative that is a carboxyatractyloside derivative.

An assay plate for high throughput screening of candidate agents that bind to at least one ANT polypeptide, comprising an assay plate having a plurality of wells, each of said wells further comprising at least one immobilized recombinant ANT polypeptide or a variant or fragment thereof.

A method of targeting a polypeptide of interest to a mitochondrial membrane, comprising expressing a recombinant expression construct encoding a fusion protein in a host cell, said construct comprising at least one regulated promoter operably linked to a nucleic acid molecule comprising a first nucleic acid sequence and a second nucleic acid sequence, said first nucleic acid sequence encoding an adenine nucleotide translocator polypeptide that is expressed as a fusion protein with a polypeptide product of said second nucleic acid sequence, wherein said second nucleic acid sequence encodes the polypeptide of interest.

A method of targeting a polypeptide of interest to a mitochondrial membrane, comprising expressing a recombinant expression construct encoding a fusion protein in a host cell, said construct comprising at least one promoter operably linked to a nucleic acid molecule comprising a first nucleic acid sequence and a second nucleic acid sequence, said first nucleic acid sequence encoding an animal adenine nucleotide translocator polypeptide that is expressed as a fusion protein with a

polypeptide product of said second nucleic acid sequence, wherein said second nucleic acid sequence encodes the polypeptide of interest; a pharmaceutical composition comprising an ANT ligand as just described.

5 A pharmaceutical composition comprising an agent that binds to an ANT polypeptide identified as just described. A pharmaceutical composition comprising an agent that binds to an ANT polypeptide identified as described above. A pharmaceutical composition comprising an agent that interacts with an ANT polypeptide identified above. A method of treatment comprising administering to a subject any one of the just described the pharmaceutical compositions.

10 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequences of the coding regions of human ANT1 ("ANT1m"), human ANT2 ("ANT2m") and human ANT3 ("ANT3m").

Figure 2 shows the polypeptide sequences of human ANT1 ("ANT1p"), human ANT2 ("ANT2p") and human ANT3 ("ANT3p").

15 Figure 3 shows induction of His-Tagged, XPRESS™-epitope containing huANT3 protein in *E. coli* as determined by Western analysis.

Figure 4 shows the localization of His-Tagged, XPRESS™-epitope containing huANT3 protein in *E. coli* as determined by Western analysis.

20 Figure 5 shows the expression of human ANT3 (huANT3) in *E. coli* expression systems.

Figure 6 shows the expression of huANT3 in baculovirus-infected Sf9 cells.

Figure 7 shows [³²P]ATP binding to Sf9/huANT3 mitochondria.

25 Figure 8 shows that ATP and atractyloside bind competitively to Sf9/huANT3 mitochondria.

Figure 9 shows high-affinity binding of atractyloside to Sf9/huANT3 mitochondria.

Figure 10 shows Northern blot analysis of huANT3 transcripts detected in a yeast expression system.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed generally toward adenine nucleotide translocator (ANT) polypeptides, which as provided herein may refer to any ANT isoform; to expression constructs containing nucleic acids encoding ANT and to natural and synthetic small molecules that interact with ANT, including ANT binding ligands. The present invention relates in part to the unexpected findings that bacterial, insect, yeast or mammalian expression systems can be designed for reliable production of recombinant human ANT polypeptides in significant quantities. In certain aspects the invention provides compositions and methods for producing recombinant ANT polypeptides that employ regulated promoters, and in certain of these and other aspects the invention provides compositions and methods for producing recombinant ANT polypeptides that are ANT fusion proteins. In certain preferred embodiments, the design of such expression systems includes the use of a host cell that lacks endogenous ANT or in which endogenous ANT gene expression is substantially impaired, as provided herein.

The present invention thus also pertains in part to methods for producing and isolating recombinant ANT polypeptides, including human ANT polypeptides and in preferred embodiments human ANT3 polypeptides, that may then be used in various binding assays and screening assays and the like. In view of the surprising observation that expression of recombinant human ANT polypeptides can be achieved at levels enabling such uses of these ANT polypeptide products, the present invention provides assays (including high throughput assays) for identifying agents that bind to recombinant human ANT. Accordingly, the present invention further relates in part to novel human ANT ligands, the synthesis, selection and characterization of which would heretofore have not been possible given the need for expressed recombinant ANT polypeptides to use in binding assays. The invention also pertains to agents that interact with ANT, including agents that enhance or impair any ANT functions known to the art, including but not limited to those described herein, and to incorporation of such agents into pharmaceutical compositions and their use in therapeutic methods.

As discussed above, the present invention relates in part to the unexpected finding that recombinant adenine nucleotide translocator (ANT) polypeptides, which includes full length ANT proteins and polypeptides, fragments and variants thereof, and further includes ANT fusion proteins as provided herein, can be produced in useful amounts by using a recombinant expression vector having a regulatory nucleic acid operably linked to a nucleic acid encoding ANT. In particular, the invention provides compositions and methods for producing recombinant ANT polypeptides through the use of a regulated promoter; the invention also provides recombinant ANT polypeptides that are ANT fusion proteins.

The invention also pertains to compositions and methods to identify the presence of ANT polypeptides and to isolate recombinant ANT, and in addition to screening assays for compounds that interact with ANT. Accordingly, the invention provides certain advantages with regard to regulation of mitochondrial function, and in particular regulation of the mitochondrial permeability “pore”. By way of background, four of the five multisubunit protein complexes (Complexes I, III, IV and V) that mediate ETC activity are localized to the inner mitochondrial membrane, which is the most protein rich of biological membranes in cells (75% by weight); the remaining ETC complex (Complex II) is situated in the matrix. ANT represents the most abundant of the inner mitochondrial membrane proteins. In at least three distinct chemical reactions known to take place within the ETC, positively-charged protons are moved from the mitochondrial matrix, across the inner membrane, to the intermembrane space. This disequilibrium of charged species creates an electrochemical potential of approximately 220 mV referred to as the “protonmotive force” (PMF), which is often represented by the notation $\Delta\psi$ or $\Delta\psi_m$ and represents the sum of the electric potential and the pH differential across the inner mitochondrial membrane (*see, e.g., Ernster et al., 1981 J. Cell Biol. 91:227s and references cited therein*).

This membrane potential drives ANT-mediated stoichiometric exchange of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) across the inner mitochondrial membrane, and provides the energy contributed to the phosphate bond created when ADP is phosphorylated to yield ATP by ETC Complex V, a process that is

“coupled” stoichiometrically with transport of a proton into the matrix. Mitochondrial membrane potential, $\Delta\psi_m$, is also the driving force for the influx of cytosolic Ca^{2+} into the mitochondrion. Under normal metabolic conditions, the inner membrane is impermeable to proton movement from the intermembrane space into the matrix, leaving ETC Complex V as the sole means whereby protons can return to the matrix. When, however, the integrity of the inner mitochondrial membrane is compromised, as occurs during MPT that may accompany a disease associated with altered mitochondrial function, protons are able to bypass the conduit of Complex V without generating ATP, thereby “uncoupling” respiration because electron transfer and associated proton pumping yields no ATP. Thus, mitochondrial permeability transition involves the opening of a mitochondrial membrane “pore”, a process by which, *inter alia*, the ETC and ATP synthesis are uncoupled, $\Delta\psi_m$ collapses and mitochondrial membranes lose the ability to selectively regulate permeability to solutes both small (*e.g.*, ionic Ca^{2+} , Na^+ , K^+ , H^+) and large (*e.g.*, proteins).

Without wishing to be bound by theory, it is unresolved whether this pore is a physically discrete conduit that is formed in mitochondrial membranes, for example by assembly or aggregation of particular mitochondrial and/or cytosolic proteins and possibly other molecular species, or whether the opening of the “pore” may simply represent a general increase in the porosity of the mitochondrial membrane.

MPT may also be induced by compounds that bind one or more mitochondrial molecular components. Such compounds include, but are not limited to, atractyloside and bongkreikic acid, which are known to bind to ANT. Methods of determining appropriate amounts of such compounds to induce MPT are known in the art (*see, e.g.*, Beutner et al., 1998 *Biochim. Biophys. Acta* 1368:7; Obatomi and Bach, 1996 *Toxicol. Lett.* 89:155; Green and Reed, 1998 *Science* 281:1309; Kroemer et al., 1998 *Annu. Rev. Physiol.* 60:619; and references cited therein). Thus certain mitochondrial molecular components, such as ANT, may contribute to the MPT mechanism. As noted above, it is believed that adenine nucleotide translocator (ANT) mediates ATP/proton exchange across the inner mitochondrial membrane, and that ANT inhibitors such as atractyloside or bongkreikic acid induce MPT under certain

conditions. Hence, it is desirable to obtain ANT in sufficient quantities for structural and functional assays that provide, for example, ANT ligands and other agents that interact with ANT, which will be useful for therapeutic management of mitochondrial pore activity. See also U.S. 09/161,172, entitled "Compositions and Methods for

5 Identifying Agents that Alter Mitochondrial Permeability Transition Pores", which is hereby incorporated by reference.

The compositions and methods of the present invention can be adapted to any prokaryotic or eukaryotic ANT, including plant and animal ANTs, which may further include, for example, yeast, vertebrate, mammalian, rodent, primate and human

10 ANTs, for which amino acid sequences and/or encoding nucleic acids will be known to those familiar with the art. Three human ANT isoforms have been described that differ in their tissue expression patterns. (Stepien et al., 1992 *J. Biol. Chem.* 267:14592; see also Wallace et al., 1998 in *Mitochondria & Free Radicals in Neurodegenerative Diseases*, Beal, Howell and Bodis-Wollner, Eds., Wiley-Liss, New York, pp. 283-307,

15 and references cited therein.) Nucleic acid sequences for cDNAs encoding these three human ANT isoforms have been reported (Figure 1; See Neckelmann et al., *Proc. Nat'l. Acad. Sci. U.S.A.* 84:7580-7584 (1987) for huANT1 cDNA [SEQ ID NO:1]; Battini et al., *J. Biol. Chem.* 262:4355-4359 (1987) for huANT2 cDNA [SEQ ID NO:2], and Cozens et al., *J. Mol. Biol.* 206:261-280 (1989) for huANT3 cDNA [SEQ ID NO:3]; see

20 Figure 2 for amino acid sequences of huANT1 [SEQ ID NO:31] huANT2 [SEQ ID NO:32] and huANT3 [SEQ ID NO:33].), and ANT gene sequences have been determined for a number of species (See, e.g., Li et al., 1989 *J. Biol. Chem.* 264:13998 for huANT1 genomic DNA; Liew et al. GenBank Acc. #N86710 for huANT2; Shinohara et al., 1993 *Biochim. Biophys. Acta* 1152:192 for rat ANT gene; for others see also, e.g., Ku et al., 1990 *J. Biol. Chem.* 265:16060; Adams et al., 1991 *Science* 252:1651; and WO 98/19714.). ANT sequences among mammalian species are highly conserved; for example, at the amino acid level murine ANT1 and ANT2 exhibit 98% sequence identity with human ANT2. Full length amino acid sequences of at least 29 ANT proteins have been reported to date from a variety of animal and plant species,

with most of these deduced from nucleic acid sequences. (Fiore et al., 1998 *Biochimie* 80:137-150)

The present invention further relates to nucleic acids which hybridize to ANT encoding polynucleotide sequences as provided herein, as incorporated by
5 reference or as will be readily apparent to those familiar with the art, if there is at least 70%, preferably at least 90%, and more preferably at least 95% identity between the sequences. The present invention particularly relates to nucleic acids which hybridize under stringent conditions to the ANT encoding nucleic acids referred to herein. As used herein, the term "stringent conditions" means hybridization will occur only if there
10 is at least 95% and preferably at least 97% identity between the sequences. The nucleic acids which hybridize to ANT encoding nucleic acids referred to herein, in preferred embodiments, encode polypeptides which either retain substantially the same biological function or activity as the ANT polypeptides encoded by the cDNAs of Figure 1 [SEQ ID NOS:1, 2 and 3], or the deposited expression constructs.

15 As used herein, to "hybridize" under conditions of a specified stringency is used to describe the stability of hybrids formed between two single-stranded nucleic acid molecules. Stringency of hybridization is typically expressed in conditions of ionic strength and temperature at which such hybrids are annealed and washed. Typically "high", "medium" and "low" stringency encompass the following conditions or
20 equivalent conditions thereto: high stringency: 0.1 x SSPE or SSC, 0.1% SDS, 65°C; medium stringency: 0.2 x SSPE or SSC, 0.1% SDS, 50°C; and low stringency: 1.0 x SSPE or SSC, 0.1% SDS, 50°C.

The deposits referred to herein will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for
25 purposes of Patent Procedure. These deposits are provided merely as convenience to those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. § 112. The sequences of the nucleic acids contained in the deposited materials, as well as the amino acid sequences of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with

any description of sequences herein. A licensee may be required to make, use or sell the deposited materials, and no such license is hereby granted.

NUCLEIC ACIDS

The nucleic acids of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. A coding sequence which encodes an ANT polypeptide for use according to the invention may be identical to the coding sequence known in the art for any given ANT, as described above and, for example, as shown for human ANT1 [SEQ ID NO:1], human ANT2 [SEQ ID NO:2] and human ANT3 [SEQ ID NO:3] in Figure 1, or to that of any deposited clone, or may be a different coding sequence, which, as a result of the redundancy or degeneracy of the genetic code, encodes the same ANT polypeptide as, for example, the cDNAs of Figure 1 or the deposited expression constructs.

The nucleic acids which encode ANT polypeptides, for example the human ANT polypeptides having the amino acid sequences of Figure 2 [SEQ ID NOS:31-33] or any other ANT polypeptides for use according to the invention, or for the ANT polypeptides encoded by the deposited constructs may include, but are not limited to: only the coding sequence for the ANT polypeptide; the coding sequence for the ANT polypeptide and additional coding sequence; the coding sequence for the ANT polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequences 5' and/or 3' of the coding sequence for the ANT polypeptide, which for example may further include but need not be limited to one or more regulatory nucleic acid sequences that may be a regulated or regulatable promoter, enhancer, other transcription regulatory sequence, repressor binding sequence, translation regulatory sequence or any other regulatory nucleic acid sequence. Thus, the term "nucleic acid encoding an ANT polypeptide" encompasses a nucleic acid which includes only coding sequence for the polypeptide as well as a nucleic acid which includes additional coding and/or non-coding sequence(s).

The present invention further relates to variants of the herein described nucleic acids which encode for fragments, analogs and derivatives of an ANT polypeptide, for example the human ANT1, ANT2 and ANT3 polypeptides having the deduced amino acid sequences of Figure 2 [SEQ ID NOS:31-33] or any ANT polypeptide, including ANT polypeptides encoded by the cDNAs of the deposited expression constructs. The variants of the nucleic acids encoding ANTs may be naturally occurring allelic variants of the nucleic acids or non-naturally occurring variants. As is known in the art, an allelic variant is an alternate form of a nucleic acid sequence which may have at least one of a substitution, a deletion or an addition of one or more nucleotides, any of which does not substantially alter the function of the encoded ANT polypeptide. Thus, for example, the present invention includes nucleic acids encoding the same ANT polypeptides as shown in Figure 2 [SEQ ID NOS:31-33], or the same ANT polypeptides encoded by the cDNAs of the deposited expression constructs, as well as variants of such nucleic acids, which variants encode a fragment, derivative or analog of any of the polypeptides of Figure 2 (SEQ ID NO:2) or the polypeptides encoded by the cDNAs of the deposited expression constructs.

Variants and derivatives of ANT may be obtained by mutations of nucleotide sequences encoding ANT polypeptides. Alterations of the native amino acid sequence may be accomplished by any of a number of conventional methods. Mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered gene wherein predetermined codons can be altered by substitution, deletion or insertion. Exemplary methods of making such alterations are disclosed by Walder et al. (*Gene* 42:133, 1986); Bauer et al. (*Gene* 37:73, 1985); Craik (*BioTechniques*, January 1985, 12-19); Smith et al. (*Genetic Engineering: Principles and Methods*, Plenum Press, 1981); Kunkel (*Proc. Natl. Acad.*

Sci. USA 82:488, 1985); Kunkel et al. (*Methods in Enzymol.* 154:367, 1987); and U.S. Patent Nos. 4,518,584 and 4,737,462.

Equivalent DNA constructs that encode various additions or substitutions of amino acid residues or sequences, or deletions of terminal or internal
 5 residues or sequences not needed for biological activity are also encompassed by the invention. For example, sequences encoding Cys residues that are not essential for biological activity can be altered to cause the Cys residues to be deleted or replaced with other amino acids, preventing formation of incorrect intramolecular disulfide bridges upon renaturation. Other equivalents can be prepared by modification of adjacent
 10 dibasic amino acid residues to enhance expression in yeast systems in which KEX2 protease activity is present. EP 212,914 discloses the use of site-specific mutagenesis to inactivate KEX2 protease processing sites in a protein. KEX2 protease processing sites are inactivated by deleting, adding or substituting residues to alter Arg-Arg, Arg-Lys, and Lys-Arg pairs to eliminate the occurrence of these adjacent basic residues. Lys-Lys
 15 pairings are considerably less susceptible to KEX2 cleavage, and conversion of Arg-Lys or Lys-Arg to Lys-Lys represents a conservative and preferred approach to inactivating KEX2 sites.

POLYPEPTIDES AND FUSION PROTEINS

The present invention further relates to ANT polypeptides, and in
 20 particular to methods for producing recombinant ANT polypeptides by culturing host cells containing ANT expression constructs, and to isolated recombinant human ANT polypeptides, including, for example, the human ANT1, ANT2 and ANT3 polypeptides which have the deduced amino acid sequence of Figure 2 [SEQ ID NOS:31-33] or which have the amino acid sequence encoded by the deposited recombinant expression
 25 constructs, as well as fragments, analogs and derivatives of such polypeptides. The polypeptides and nucleic acids of the present invention are preferably provided in an isolated form, and in certain preferred embodiments are purified to homogeneity.

The terms "fragment," "derivative" and "analog" when referring to ANT polypeptides or fusion proteins, or to ANT polypeptides or fusion proteins encoded by

the deposited recombinant expression constructs, refers to any ANT polypeptide or fusion protein that retains essentially the same biological function or activity as such polypeptide. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active ANT polypeptide. The polypeptide of the present invention may be a recombinant polypeptide or a synthetic polypeptide, and is preferably a recombinant polypeptide.

A fragment, derivative or analog of an ANT polypeptide or fusion protein, including ANT polypeptides or fusion proteins encoded by the cDNAs of the deposited constructs, may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the ANT polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which additional amino acids are fused to the ANT polypeptide, including amino acids that are employed for purification of the ANT polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides of the present invention include ANT polypeptides and fusion proteins having amino acid sequences that are identical or similar to sequences known in the art. For example by way of illustration and not limitation, the human ANT ("huANT") polypeptides of Figure 2 [SEQ ID NOS:31-33] are contemplated for use according to the instant invention, as are polypeptides having at least 70% similarity (preferably a 70% identity) to the polypeptides of Figure 2 [SEQ ID NOS:31-33] and more preferably 90% similarity (more preferably a 90% identity) to the polypeptides of Figure 2 [SEQ ID NOS: 31-33] and still more preferably a 95% similarity (still more preferably a 95% identity) to the polypeptides of Figure 2 [SEQ ID NOS:31-33] and to portions of such polypeptides, wherein such portions of an ANT polypeptide generally contain at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art “similarity” between two polypeptides is determined by comparing the amino acid sequence and conserved amino acid substitutes thereto of the polypeptide to the sequence of a second polypeptide. Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length polypeptides. Fragments or portions of the nucleic acids of the present invention may be used to synthesize full-length nucleic acids of the present invention.

The term “isolated” means that the material is removed from its original environment (*e.g.*, the natural environment if it is naturally occurring). For example, a naturally occurring nucleic acid or polypeptide present in a living animal is not isolated, but the same nucleic acid or polypeptide, separated from some or all of the co-existing materials in the natural system, is isolated. Such nucleic acids could be part of a vector and/or such nucleic acids or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The term “gene” means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region “leader and trailer” as well as intervening sequences (introns) between individual coding segments (exons).

As described herein, the invention provides ANT fusion proteins encoded by nucleic acids that have the ANT coding sequence fused in frame to an additional coding sequence to provide for expression of an ANT polypeptide sequence fused to an additional functional or non-functional polypeptide sequence that permits, for example by way of illustration and not limitation, detection, isolation and/or purification of the ANT fusion protein. Such ANT fusion proteins may permit detection, isolation and/or purification of the ANT fusion protein by protein-protein affinity, metal affinity or charge affinity-based polypeptide purification, or by specific protease cleavage of a fusion protein containing a fusion sequence that is cleavable by a protease such that the ANT polypeptide is separable from the fusion protein.

Thus ANT fusion proteins may comprise polypeptide sequences added to ANT to facilitate detection and isolation of ANT. Such peptides include, for example, poly-His or the antigenic identification peptides described in U.S. Patent No. 5,011,912 and in Hopp et al., (1988 *Bio/Technology* 6:1204), or the XPRESS™ epitope tag
 5 (Invitrogen, Carlsbad, CA). The affinity sequence may be a hexa-histidine tag as supplied, for example, by a pBAD/His (Invitrogen) or a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or, for example, the affinity sequence may be a hemagglutinin (HA) tag when a mammalian host, *e.g.*, COS-7 cells, is used. The HA tag corresponds to an antibody
 10 defined epitope derived from the influenza hemagglutinin protein (Wilson et al., *Cell* 37:767 (1984)).

ANT fusion proteins may further comprise immunoglobulin constant region polypeptides added to ANT to facilitate detection, isolation and/or localization of ANT. The immunoglobulin constant region polypeptide preferably is fused to the C-
 15 terminus of an ANT polypeptide. General preparation of fusion proteins comprising heterologous polypeptides fused to various portions of antibody-derived polypeptides (including the Fc domain) has been described, *e.g.*, by Ashkenazi et al. (*PNAS USA* 88:10535, 1991) and Byrn et al. (*Nature* 344:677, 1990). A gene fusion encoding the ANT:Fc fusion protein is inserted into an appropriate expression vector. In certain
 20 embodiments of the invention, ANT:Fc fusion proteins may be allowed to assemble much like antibody molecules, whereupon interchain disulfide bonds form between Fc polypeptides, yielding dimeric ANT fusion proteins.

ANT fusion proteins having specific binding affinities for pre-selected antigens by virtue of fusion polypeptides comprising immunoglobulin V-region
 25 domains encoded by DNA sequences linked in-frame to sequences encoding ANT are also within the scope of the invention, including variants and fragments thereof as provided herein. General strategies for the construction of fusion proteins having immunoglobulin V-region fusion polypeptides are disclosed, for example, in EP 0318554; U.S. 5,132,405; U.S. 5,091,513; and U.S. 5,476,786.

The nucleic acid of the present invention may also encode a fusion protein comprising an ANT polypeptide fused to other polypeptides having desirable affinity properties, for example an enzyme such as glutathione-S-transferase. As another example, ANT fusion proteins may also comprise an ANT polypeptide fused to a *Staphylococcus aureus* protein A polypeptide; protein A encoding nucleic acids and their use in constructing fusion proteins having affinity for immunoglobulin constant regions are disclosed generally, for example, in U.S. Patent 5,100,788. Other useful affinity polypeptides for construction of ANT fusion proteins may include streptavidin fusion proteins, as disclosed, for example, in WO 89/03422; U.S. 5,489,528; U.S. 5,672,691; WO 93/24631; U.S. 5,168,049; U.S. 5,272,254 and elsewhere, and avidin fusion proteins (see, *e.g.*, EP 511,747). As provided herein and in the cited references, ANT polypeptide sequences may be fused to fusion polypeptide sequences that may be full length fusion polypeptides and that may alternatively be variants or fragments thereof.

The present invention also provides a method of targeting a polypeptide of interest to a membrane, and in particular embodiments to a cellular membrane, and in further embodiments to a mitochondrial membrane. This aspect of the invention is based on the unexpected observation that certain recombinant expression constructs as provided herein, which constructs include a nucleic acid encoding a first polypeptide that is an ANT polypeptide, and that is expressed as a fusion protein with a second polypeptide sequence, provide recombinant ANT fusion proteins capable of preferentially localizing to cell membranes. In certain embodiments the cell membrane is a prokaryotic cell membrane such as a bacterial cell membrane, for example an *E. coli* membrane. In other embodiments the cell membrane is a eukaryotic cell membrane such as a yeast or a mammalian cell membrane, for example a membrane of any eukaryotic cell contemplated herein.

A cell membrane as used herein may be any cellular membrane, and typically refers to membranes that are in contact with cytosolic components, including intracellular membrane bounded compartments such as mitochondrial inner and outer membranes as described above, and also intracellular vesicles, ER-Golgi constituents,

other organelles and the like, as well as the plasma membrane. In preferred embodiments, an ANT fusion protein may be targeted to a mitochondrial membrane. In other preferred embodiments, for example, recombinant expression constructs according to the invention may encode ANT fusion proteins that contain polypeptide sequences that direct the fusion protein to be retained in the cytosol, to reside in the lumen of the endoplasmic reticulum (ER), to be secreted from a cell via the classical ER-Golgi secretory pathway, to be incorporated into the plasma membrane, to associate with a specific cytoplasmic component including the cytoplasmic domain of a transmembrane cell surface receptor or to be directed to a particular subcellular location by any of a variety of known intracellular protein sorting mechanisms with which those skilled in the art will be familiar. Accordingly, these and related embodiments are encompassed by the instant compositions and methods directed to targeting a polypeptide of interest to a predefined intracellular, membrane or extracellular localization.

15 VECTORS

The present invention also relates to vectors and to constructs that include nucleic acids of the present invention, and in particular to “recombinant expression constructs” that include any nucleic acids encoding ANT polypeptides according to the invention as provided above; to host cells which are genetically engineered with vectors and/or constructs of the invention and to the production of ANT polypeptides and fusion proteins of the invention, or fragments or variants thereof, by recombinant techniques. ANT proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor, NY, (1989).

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, *e.g.*, the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α -factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, *e.g.*, stabilization or simplified purification of expressed recombinant product.

Useful expression constructs for bacterial use are constructed by inserting into an expression vector a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The construct may comprise one or more phenotypic selectable marker and an origin of replication to ensure maintenance of the vector construct and, if desirable, to provide amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium* and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although others may also be employed as a matter of choice. Any other plasmid or vector may be used as long as they are replicable and viable in the host.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 “backbone” sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter, if it is a regulated promoter

as provided herein, is induced by appropriate means (*e.g.*, temperature shift or chemical induction) and cells are cultured for an additional period. Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification. Microbial cells employed in expression of

5 proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents; such methods are well known to those skilled in the art.

Thus, for example, the nucleic acids of the invention as provided herein may be included in any one of a variety of expression vector constructs as a

10 recombinant expression construct for expressing an ANT polypeptide. Such vectors and constructs include chromosomal, nonchromosomal and synthetic DNA sequences, *e.g.*, derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA, such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may

15 be used for preparation of a recombinant expression construct as long as it is replicable and viable in the host.

The appropriate DNA sequence(s) may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Standard techniques for

20 cloning, DNA isolation, amplification and purification, for enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like, and various separation techniques are those known and commonly employed by those skilled in the art. A number of standard techniques are described, for example, in Ausubel et al. (1993 *Current Protocols in Molecular Biology*, Greene Publ. Assoc. Inc.

25 & John Wiley & Sons, Inc., Boston, MA); Sambrook et al. (1989 *Molecular Cloning*, Second Ed., Cold Spring Harbor Laboratory, Plainview, NY); Maniatis et al. (1982 *Molecular Cloning*, Cold Spring Harbor Laboratory, Plainview, NY); and elsewhere.

The DNA sequence in the expression vector is operatively linked to at least one appropriate expression control sequences (*e.g.*, a promoter or a regulated

30 promoter) to direct mRNA synthesis. Representative examples of such expression

control sequences include LTR or SV40 promoter, the *E. coli lac* or *trp*, the phage lambda P_L promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with
 5 selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P_R, P_L and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art, and
 10 preparation of certain particularly preferred recombinant expression constructs comprising at least one promoter or regulated promoter operably linked to a nucleic acid encoding an ANT polypeptide is described herein.

In certain preferred embodiments the expression control sequence is a “regulated promoter”, which may be a promoter as provided herein and may also be a
 15 repressor binding site, an activator binding site or any other regulatory sequence that controls expression of a nucleic acid sequence as provided herein. In certain particularly preferred embodiments the regulated promoter is a tightly regulated promoter that is specifically inducible and that permits little or no transcription of nucleic acid sequences under its control in the absence of an induction signal, as is
 20 known to those familiar with the art and described, for example, in Guzman et al. (1995 *J. Bacteriol.* 177:4121), Carra et al. (1993 *EMBO J.* 12:35), Mayer (1995 *Gene* 163:41), Haldimann et al. (1998 *J. Bacteriol.* 180:1277), Lutz et al. (1997 *Nuc. Ac. Res.* 25:1203), Allgood et al. (1997 *Curr. Opin. Biotechnol.* 8:474) and Makrides (1996 *Microbiol. Rev.* 60:512), all of which are hereby incorporated by reference. In other
 25 preferred embodiments of the invention a regulated promoter is present that is inducible but that may not be tightly regulated. In certain other preferred embodiments a promoter is present in the recombinant expression construct of the invention that is not a regulated promoter; such a promoter may include, for example, a constitutive promoter such as an insect polyhedrin promoter as described in the Examples or a yeast
 30 phosphoglycerate kinase promoter (see, *e.g.*, Giraud et al., 1998 *J. Mol. Biol.* 281:409).

The expression construct also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are *cis*-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

As noted above, in certain embodiments the vector may be a viral vector such as a retroviral vector. For example, retroviruses from which the retroviral plasmid vectors may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus.

The viral vector includes one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., *Biotechniques* 7:980-990 (1989), or any other promoter (*e.g.*, cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and β -actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein, and may be from among either regulated promoters or promoters as described above.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, ψ -2, ψ -AM, PA12, T19-14X, VT-19-17-H2, ψ CRE, ψ CRIP, GP+E-86, GP+envAm12, and DAN cell lines as

described in Miller, *Human Gene Therapy*, 1:5-14 (1990), which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and CaPO₄ precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

The producer cell line generates infectious retroviral vector particles which include the nucleic acid sequence(s) encoding the ANT polypeptides or fusion proteins. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either *in vitro* or *in vivo*. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the ANT polypeptide or fusion protein. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

As another example of an embodiment of the invention in which a viral vector is used to prepare the recombinant ANT expression construct, in one preferred embodiment, host cells transduced by a recombinant viral construct directing the expression of ANT polypeptides or fusion proteins may produce viral particles containing expressed ANT polypeptides or fusion proteins that are derived from portions of a host cell membrane incorporated by the viral particles during viral budding. In another preferred embodiment, ANT encoding nucleic acid sequences are cloned into a baculovirus shuttle vector, which is then recombined with a baculovirus to generate a recombinant baculovirus expression construct that is used to infect, for example, Sf9 host cells, as described in *Baculovirus Expression Protocols, Methods in Molecular Biology* Vol. 39, Christopher D. Richardson, Editor, Human Press, Totowa, NJ, 1995; Piwnica-Worms, "Expression of Proteins in Insect Cells Using Baculoviral Vectors," Section II in Chapter 16 in: *Short Protocols in Molecular Biology*, 2nd Ed., Ausubel *et al.*, eds., John Wiley & Sons, New York, New York, 1992, pages 16-32 to 16-48.

HOST CELLS

In another aspect, the present invention relates to host cells containing the above described recombinant ANT expression constructs. Host cells are genetically engineered (transduced, transformed or transfected) with the vectors and/or expression constructs of this invention which may be, for example, a cloning vector, a shuttle vector or an expression construct. The vector or construct may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying particular genes such as genes encoding ANT polypeptides or ANT fusion proteins. The culture conditions for particular host cells selected for expression, such as temperature, pH and the like, will be readily apparent to the ordinarily skilled artisan.

The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Representative examples of appropriate host cells according to the present invention include, but need not be limited to, bacterial cells, such as *E. coli*, *Streptomyces*, *Salmonella typhimurium*; fungal cells, such as yeast; insect cells, such as *Drosophila S2* and *Spodoptera Sf9*; animal cells, such as CHO, COS or 293 cells; adenoviruses; plant cells, or any suitable cell already adapted to *in vitro* propagation or so established *de novo*. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, *Cell* 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences, for example as described herein regarding the preparation of ANT expression constructs. DNA

sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements. Introduction of the construct into the host cell can be effected by a variety of methods with which those skilled in the art will be familiar, including but not limited to, for example, calcium phosphate
 5 transfection, DEAE-Dextran mediated transfection, or electroporation (Davis et al., 1986 *Basic Methods in Molecular Biology*).

As will be appreciated by those of ordinary skill in the art, in certain situations it may be desirable to prepare the compositions of the invention and to practice the methods of the invention under conditions where endogenous ANT
 10 expression by a host cell is compromised, in order to provide advantages associated with the expression of a desired ANT encoding construct. For example, detection of particular ANT encoding nucleic acid sequences or ANT polypeptides that are highly similar to those encoded by the host cell genome may be facilitated by inhibiting host cell ANT gene expression. As another example, where functional activity of an
 15 exogenously introduced recombinant ANT polypeptide is to be determined in a host cell or in a biological sample derived therefrom, it may also be advantageous to inhibit endogenous host cell ANT gene expression.

Thus, in certain preferred embodiments of the invention, host cells may lack at least one isoform of an endogenous ANT, and in certain preferred embodiments
 20 the host cells may lack all endogenous ANT isoforms. For example, in the yeast system described by Giraud et al. (1998 J. Mol. Biol. 281:409) a *S. cerevisiae* triple null mutant is described that lacks all three yeast ANT isoforms and is unable to grow under anaerobic conditions. In other preferred embodiments, expression in host cells of at least one gene encoding an endogenous ANT isoform is substantially impaired.
 25 Substantial impairment of endogenous ANT isoform expression may be achieved by any of a variety of methods that are well known in the art for blocking specific gene expression, including site-specific or site-directed mutagenesis as described above, antisense inhibition of gene expression, ribozyme mediated inhibition of gene expression and generation of mitochondrial DNA depleted (ρ^0) cells.

Identification of oligonucleotides and ribozymes for use as antisense agents and DNA encoding genes for targeted delivery for genetic therapy involve methods well known in the art. For example, the desirable properties, lengths and other characteristics of such oligonucleotides are well known. Antisense oligonucleotides are typically designed to resist degradation by endogenous nucleolytic enzymes by using such linkages as: phosphorothioate, methylphosphonate, sulfone, sulfate, ketyl, phosphorodithioate, phosphoramidate, phosphate esters, and other such linkages (*see, e.g., Agrwal et al., Tetrahedron Lett.* 28:3539-3542 (1987); Miller et al., *J. Am. Chem. Soc.* 93:6657-6665 (1971); Stec et al., *Tetrahedron Lett.* 26:2191-2194 (1985); Moody et al., *Nucl. Acids Res.* 12:4769-4782 (1989); Uznanski et al., *Nucl. Acids Res.* (1989); Letsinger et al., *Tetrahedron* 40:137-143 (1984); Eckstein, *Annu. Rev. Biochem.* 54:367-402 (1985); Eckstein, *Trends Biol. Sci.* 14:97-100 (1989); Stein In: *Oligodeoxynucleotides. Antisense Inhibitors of Gene Expression*, Cohen, Ed, Macmillan Press, London, pp. 97-117 (1989); Jager et al., *Biochemistry* 27:7237-7246 (1988)).

Antisense nucleotides are oligonucleotides that bind in a sequence-specific manner to nucleic acids, such as mRNA or DNA. When bound to mRNA that has complementary sequences, antisense prevents translation of the mRNA (*see, e.g., U.S. Patent No. 5,168,053 to Altman et al.; U.S. Patent No. 5,190,931 to Inouye, U.S. Patent No. 5,135,917 to Burch; U.S. Patent No. 5,087,617 to Smith and Clusel et al.* (1993) *Nucl. Acids Res.* 21:3405-3411, which describes dumbbell antisense oligonucleotides). Triplex molecules refer to single DNA strands that bind duplex DNA forming a colinear triplex molecule, thereby preventing transcription (*see, e.g., U.S. Patent No. 5,176,996 to Hogan et al., which describes methods for making synthetic oligonucleotides that bind to target sites on duplex DNA*).

According to this embodiment of the invention, particularly useful antisense nucleotides and triplex molecules are molecules that are complementary to or bind the sense strand of DNA or mRNA that encodes an ANT polypeptide or a protein mediating any other process related to expression of endogenous ANT genes, such that inhibition of translation of mRNA encoding the ANT polypeptide is effected.

A ribozyme is an RNA molecule that specifically cleaves RNA substrates, such as mRNA, resulting in specific inhibition or interference with cellular gene expression. There are at least five known classes of ribozymes involved in the cleavage and/or ligation of RNA chains. Ribozymes can be targeted to any RNA transcript and can catalytically cleave such transcripts (*see, e.g.*, U.S. Patent No. 5,272,262; U.S. Patent No. 5,144,019; and U.S. Patent Nos. 5,168,053, 5,180,818, 5,116,742 and 5,093,246 to Cech et al.). According to certain embodiments of the invention, any such ANT mRNA-specific ribozyme, or a nucleic acid encoding such a ribozyme, may be delivered to a host cell to effect inhibition of ANT gene expression.

10 Ribozymes, and the like may therefore be delivered to the host cells by DNA encoding the ribozyme linked to a eukaryotic promoter, such as a eukaryotic viral promoter, such that upon introduction into the nucleus, the ribozyme will be directly transcribed.

As used herein, expression of a gene encoding an endogenous adenine nucleotide translocator isoform is substantially impaired by any of the above methods for inhibiting when cells are substantially but not necessarily completely depleted of functional DNA or functional mRNA encoding the endogenous ANT isoform, or of the relevant ANT polypeptide. ANT isoform expression is substantially impaired when cells are preferably at least 50% depleted of DNA or mRNA encoding the endogenous ANT (as measured using high stringency hybridization as described above) or depleted

15 of ANT polypeptide (as measured by Western immunoblot as described herein, see also, *e.g.*, Giraud et al. 1998 *J. Mol Biol.* 281:409); and more preferably at least 75% depleted of endogenous ANT DNA, mRNA or polypeptide. Most preferably, ANT isoform expression is substantially impaired when cells are depleted of >90% of their endogenous ANT DNA, mRNA, or polypeptide.

25 Alternatively, expression of a gene encoding an endogenous adenine nucleotide translocator isoform may be substantially impaired through the use of mitochondrial DNA depleted ρ^0 cells, which are incapable of mitochondrial replication and so may not continue to express functional ANT polypeptides. Methods for producing ρ^0 cells are known and can be found, for example in PCT/US95/04063,

30 which is hereby incorporated by reference.

PROTEIN PRODUCTION

The expressed recombinant ANT polypeptides or fusion proteins may be useful in intact host cells; in intact organelles such as mitochondria, cell membranes, intracellular vesicles other cellular organelles; or in disrupted cell preparations including but not limited to cell homogenates or lysates, submitochondrial particles, uni- and multilamellar membrane vesicles or other preparations. Alternatively, expressed recombinant ANT polypeptides or fusion proteins can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

SAMPLES

A “biological sample containing mitochondria” may comprise any tissue or cell preparation in which intact mitochondria capable of maintaining a membrane potential when supplied with one or more oxidizable substrates such as glucose, malate or galactose are or are thought to be present. Mitochondrial membrane potential may be determined according to methods with which those skilled in the art will be readily familiar, including but not limited to detection and/or measurement of detectable compounds such as fluorescent indicators, optical probes and/or sensitive pH and ion-

selective electrodes (*See, e.g.,* Ernster et al., 1981 *J. Cell Biol.* 91:227s and references cited therein; *see also* Haugland, 1996 *Handbook of Fluorescent Probes and Research Chemicals- Sixth Ed.*, Molecular Probes, Eugene, OR, pp. 266-274 and 589-594.). By “capable of maintaining a potential” it is meant that such mitochondria have a

5 membrane potential that is sufficient to permit the accumulation of a detectable compound (*e.g.,* DASPMI [2-,4-dimethylaminostyryl-N-methylpyridinium], TMRM [tetramethylrhodamine methyl ester], *etc.*) used in the particular instance. A biological sample containing mitochondria may, for example, be derived from a normal (*i.e.,* healthy) individual or from an individual having a disease associated with altered

10 mitochondrial function. Biological samples containing mitochondria may be provided by obtaining a blood sample, biopsy specimen, tissue explant, organ culture or any other tissue or cell preparation from a subject or a biological source. The subject or biological source may be a human or non-human animal, a primary cell culture or culture adapted cell line including but not limited to genetically engineered cell lines

15 that may contain chromosomally integrated or episomal recombinant nucleic acid sequences, immortalized or immortalizable cell lines, somatic cell hybrid or cytoplasmic hybrid “cybrid” cell lines, differentiated or differentiable cell lines, transformed cell lines and the like.

A “biological sample” may comprise any tissue or cell preparation as just

20 described for a biological sample containing mitochondria, but does not require the presence of intact mitochondria. Thus a “biological sample” may comprise any tissue or cell preparation and a “biological sample containing at least one recombinant ANT polypeptide” comprises any tissue or cell preparation in which an expressed recombinant ANT polypeptide or fusion protein as provided herein is thought to be

25 present. A biological sample may, for example, be derived from a recombinant cell line or from a transgenic animal. Biological samples containing recombinant ANT may be provided by obtaining a blood sample, biopsy specimen, tissue explant, organ culture or any other tissue or cell preparation from a subject or a biological source. The subject or biological source may be a human or non-human animal, a primary cell culture or

30 culture adapted cell line including but not limited to genetically engineered cell lines

that may contain chromosomally integrated or episomal recombinant nucleic acid sequences, immortalized or immortalizable cell lines, somatic cell hybrid or cytoplasmic hybrid “cybrid” cell lines, differentiated or differentiable cell lines, transformed cell lines and the like.

5 PROTEINS

As described herein, isolation of a mitochondrial pore component or a mitochondrial molecular species with which an agent identified according to the methods of the invention interacts refers to physical separation of such a complex from its biological source, and may be accomplished by any of a number of well known techniques including but not limited to those described herein, and in the cited references. Without wishing to be bound by theory, a compound that “binds a mitochondrial component” can be any discrete molecule, agent compound, composition of matter or the like that may, but need not, directly bind to a mitochondrial molecular component, and may in the alternative bind indirectly to a mitochondrial molecular component by interacting with one or more additional components that bind to a mitochondrial molecular component. These or other mechanisms by which a compound may bind to and/or associate with a mitochondrial molecular component are within the scope of the claimed methods, so long as isolating a mitochondrial pore component also results in isolation of the mitochondrial molecular species that directly or indirectly binds to the identified agent. Thus, for example, as provided herein, any ANT polypeptide including recombinant ANT polypeptides and fusion proteins may be a mitochondrial molecular component and/or a mitochondrial pore component, and any ANT ligand or agent that binds to an ANT polypeptide may be a compound that binds a mitochondrial component and/or an agent that affects mitochondrial pore activity.

As described herein, the mitochondrial permeability transition “pore” may not be a discrete assembly or multisubunit complex, and the term thus refers instead to any mitochondrial molecular component (including, *e.g.*, a mitochondrial membrane *per se*) that regulates the inner membrane selective permeability where such regulated function is impaired during MPT. As used herein, mitochondria are

comprised of “mitochondrial molecular components”, which may be any protein, polypeptide, peptide, amino acid, or derivative thereof; any lipid, fatty acid or the like, or derivative thereof; any carbohydrate, saccharide or the like or derivative thereof, any nucleic acid, nucleotide, nucleoside, purine, pyrimidine or related molecule, or derivative thereof, or the like; or any other biological molecule that is a constituent of a mitochondrion. “Mitochondrial molecular components” includes but is not limited to “mitochondrial pore components”. A “mitochondrial pore component” is any mitochondrial molecular component that regulates the selective permeability characteristic of mitochondrial membranes as described above, including those responsible for establishing $\Delta\Psi_m$ and those that are functionally altered during MPT.

Isolation and, optionally, identification and/or characterization of the mitochondrial pore component or components with which an agent that affects mitochondrial pore activity interacts may also be desirable and are within the scope of the invention. Once an agent is shown to alter MPT according to the methods provided herein and in U.S. 09/161,172, those having ordinary skill in the art will be familiar with a variety of approaches that may be routinely employed to isolate the molecular species specifically recognized by such an agent and involved in regulation of MPT, where to “isolate” as used herein refers to separation of such molecular species from the natural biological environment. Thus, for example, once an ANT ligand is prepared according to the methods provided herein, such approaches may be routinely employed to isolate the ANT polypeptide. Techniques for isolating a mitochondrial pore component such as an ANT polypeptide or fusion protein may include any biological and/or biochemical methods useful for separating the complex from its biological source, and subsequent characterization may be performed according to standard biochemical and molecular biology procedures. Those familiar with the art will be able to select an appropriate method depending on the biological starting material and other factors. Such methods may include, but need not be limited to, radiolabeling or otherwise detectably labeling cellular and mitochondrial components in a biological sample, cell fractionation, density sedimentation, differential extraction, salt precipitation, ultrafiltration, gel filtration, ion-exchange chromatography, partition

chromatography, hydrophobic chromatography, electrophoresis, affinity techniques or any other suitable separation method that can be adapted for use with the agent with which the mitochondrial pore component interacts. Antibodies to partially purified components may be developed according to methods known in the art and may be used to detect and/or to isolate such components.

Affinity techniques may be particularly useful in the context of the present invention, and may include any method that exploits a specific binding interaction between a mitochondrial pore component and an agent identified according to the invention that interacts with the pore component. For example, because ANT ligands as provided herein and other agents that influence MPT can be immobilized on solid phase matrices, an affinity binding technique for isolation of the pore component may be particularly useful. Alternatively, affinity labeling methods for biological molecules, in which a known MPT-active agent or a novel ANT ligand as provided herein may be modified with a reactive moiety, are well known and can be readily adapted to the interaction between the agent and a pore component, for purposes of introducing into the pore component a detectable and/or recoverable labeling moiety. (See, e.g., *Pierce Catalog and Handbook*, 1994 Pierce Chemical Company, Rockford, IL; Scopes, R.K., *Protein Purification: Principles and Practice*, 1987, Springer-Verlag, New York; and Hermanson, G.T. et al., *Immobilized Affinity Ligand Techniques*, 1992, Academic Press, Inc., California; for details regarding techniques for isolating and characterizing biological molecules, including affinity techniques.

Characterization of mitochondrial pore component molecular species, isolated by MPT-active agent affinity techniques described above or by other biochemical methods, may be accomplished using physicochemical properties of the pore component such as spectrometric absorbance, molecular size and/or charge, solubility, peptide mapping, sequence analysis and the like. (See, e.g., Scopes, *supra*.) Additional separation steps for biomolecules may be optionally employed to further separate and identify molecular species that co-purify with mitochondrial pore components. These are well known in the art and may include any separation methodology for the isolation of proteins, lipids, nucleic acids or carbohydrates,

typically based on physicochemical properties of the newly identified components of the complex. Examples of such methods include RP-HPLC, ion exchange chromatography, hydrophobic interaction chromatography, hydroxyapatite chromatography, native and/or denaturing one- and two-dimensional electrophoresis, ultrafiltration, capillary electrophoresis, substrate affinity chromatography, immunoaffinity chromatography, partition chromatography or any other useful separation method. Preferably extracts of cultured cells, and in particularly preferred embodiments extracts of biological tissues or organs may be sources of mitochondrial molecular components, including ANT polypeptides. Preferred sources may include blood, brain, fibroblasts, myoblasts, liver cells or other cell types.

ANT LIGANDS

As noted above, the binding of the adenine nucleotide translocator (ANT) is responsible for mediating transport of ADP and ATP across the mitochondrial inner membrane. ANT has also been implicated as the critical component of the mitochondrial permeability transition pore, a Ca^{2+} regulated inner membrane channel that plays an important modulating role in apoptotic processes. Additionally, ANT activity appears to be related to changes in ANT polypeptide conformation within the mitochondrial membrane, as evidenced by studies using agents that are capable of binding to ANT. (Block et al., 1986 *Meths. Enzymol.* 125:658) Accordingly, it is another aspect of the present invention to provide compositions and methods for producing and identifying agents that bind to ANT, which agents are also referred to herein as ANT ligands.

Binding interactions between ANT and a variety of small molecules are known to those familiar with the art. For example, these interactions include binding to ANT by atractyloside, carboxyatractyloside, palmitoyl-CoA, bongkreikic acid, thyroxin, eosin Y and erythrosin B. (See, e.g., Stubbs, 1979 *Pharm. Ther.* 7:329; Klingenberg et al., 1978 *Biochim. Biophys. Acta* 503:193; Sterling, 1986 *Endocrinol.* 119:292; Majima et al., 1998 *Biochem.* 37:424; Block et al. 1986 *Meths. Enzymol.* 125:658; for erythrosin

B and additional ANT inhibitors, see Beavis et al. 1993 *J. Biol. Chem.* 268:997; Powers et al. 1994 *J. Biol. Chem.* 269:10614.)

The ANT ligands of the present invention represent novel atractyloside derivatives. Atractyloside (ATR) and its known derivatives, including carboxyatractyloside (CATR), naphthoyl-ATR, MANT-ATR and other ATR derivatives (see, e.g., Boulay et al., *Analytical Biochemistry* 128:323-330,1983; Roux et al., *Analytical Biochemistry* 234:31-37,1996; Lauquin et al., *FEBS Letters* 67:306-311,1976; and Gottikh et al., *Tetrahedron* 26:4419-4433, 1970; for other known ATR derivatives see, e.g., Block et al., 1986 *Meths. Enzymol.* 125:658) have proven invaluable in the elucidation of the structure and the mechanism of action of the adenine nucleotide translocator. According to the ANT ligands of the invention, the binding mode of ATR to ANT allows for modifications of the ATR 6'-hydroxyl functionality without significantly altering ATR binding affinity for ANT. Thus, ANT ligands as provided herein may be ATR derivatives modified by chemical substitution at the 6' hydroxyl position. In particular, the novel ANT ligands as provided herein further include long linker moieties at the 6' position, which linkers may include a 6'-amine linker, thereby permitting additional chemical modification to the ANT ligand as will be appreciated by those skilled in the art and as illustrated in the non-limiting Examples. Also, as shown in Examples 6-9, such linkers as provided herein may have carbon chain backbones of 2-20 carbon atoms, and in preferred embodiments 2-6 carbon atoms.

The invention therefore provides ANT ligands that may be intermediates for conjugation to a variety of additional chemical moieties to yield further ATR derivatives that are ANT ligands within the scope of the invention. These include ANT ligands to which ^{125}I may be covalently attached under mild reaction conditions; the invention also includes ANT ligands to which reactive amine groups may be covalently linked. ANT ligands which are such amine-containing ATR derivatives may then be reacted with a variety of fluorophores and haptens bearing, for example, reactive isothiocyanate, N-hydroxysuccinimide ester, anhydride and other useful functionalities to yield stable ATR derivatives including, for example, derivatives that have thiourea, amide or other linkages.

Thus, ANT ligands as provided herein also include ATR derivatives that are detectable by virtue of substituents introduced at the 6' position. Accordingly, detectable ATR derivatives as herein provided include ATR derivatives having a 6' hydroxyl substitution that includes a radiolabeled substituent, for example ^{125}I , ^{131}I , ^3H , ^{14}C or ^{35}S . Other ANT ligands that are detectable ATR derivatives may comprise fluorescent substituents, including those appropriately tagged with reporter molecules such as fluorophores and haptens having utility in high throughput screening assays for identifying agents that bind to ANT. More specifically, in preferred embodiments, an ANT ligand according to the present invention that includes fluorescent substituents has an extinction coefficient $\geq 10,000 \text{ M}^{-1}$ (see Table 1); further, this property provides an advantage for using such ANT ligands according to the methods provided herein, and in particular for use in high throughput screening assays. Additionally, the ANT ligands of the invention exhibit high affinities for ANT, and in preferred embodiments have binding constants in the nanomolar range.

In certain embodiments of the invention, ANT ligands may be ATR derivatives such as ATR-lanthanide chelating agents, which have utility in time-resolved fluorescence detection, for example detection of these compounds complexed to a lanthanide ion such as Eu^{3+} , Tb^{3+} , Sm^{3+} and Dy^{3+} . In addition, ANT ligands may comprise ATR conjugated to readily detectable substituents such as highly fluorescent moieties, for example by way of illustration and not limitation, cyanine and coumarin. These and other highly fluorescent substituents permit the synthesis, according to the methods of the invention, of ANT ligands that are detectable with extremely high sensitivities. Those familiar with the art are aware of additional fluorescent substituents that may be used, for example, those disclosed in Haugland, 1996 *Handbook of Fluorescent Probes and Research Chemicals- Sixth Ed.*, Molecular Probes, Eugene, OR. In other embodiments, the invention provides detectable ANT ligands produced by coupling of biotin-NHS ester with the ATR derivatives as disclosed herein; these and other ANT ligands similarly generated according to the instant methods can be detected with commercially available enzyme-avidin conjugates using, for example, colorimetric, fluorescent or chemiluminescent techniques.

ASSAYS

It is another aspect of the invention to provide compositions and methods for the determination of the presence of ANT polypeptides and for the identification of agents that bind to, or that interact with, ANT polypeptides. Such compositions and methods will be useful for diagnostic and prognostic purposes, for example in the determination of the existence of altered mitochondrial function which, as described above, may accompany both normal and disease states. These compositions and methods will also be useful for the identification of agents that alter or regulate mitochondrial function based on ANT roles in mitochondrial activities, for example by way of illustration and not limitation, maintenance of mitochondrial membrane potential, ATP biosynthesis, induction of apoptosis, MPT and other mitochondrial function. In certain preferred embodiments these compositions and methods are useful as high throughput screening assays.

In certain aspects the invention provides a method for determining the presence of an ANT polypeptide in a biological sample, comprising contacting a sample suspected of containing an ANT polypeptide with an ANT ligand under conditions and for a time sufficient to allow binding of the ANT ligand to an ANT polypeptide, and detecting such binding. "ANT ligands" according to these aspects of the invention may include any novel ANT ligands as provided herein. The use of human ANT1, ANT2 and ANT3 according to these methods represent particularly preferred embodiments. Other preferred embodiments include the use of any ANT polypeptide or ANT fusion protein as provided herein. Accordingly, the instant method for determining the presence of ANT polypeptide in a sample will be useful for monitoring expression of ANT encoding constructs provided herein. In some preferred embodiments an ANT fusion protein is used that is a GST fusion protein, and in other preferred embodiments the ANT fusion protein is a His-tagged fusion protein. As provided herein, the biological sample may be a cell, a mitochondrion, submitochondrial particles, a cell membrane (including any cellular membrane as described herein), a cell extract, cell conditioned medium, a tissue homogenate or an isolated ANT.

In other aspects, the invention provides a method for identifying an agent that binds to an ANT polypeptide, comprising contacting a candidate agent with a host cell expressing at least one recombinant ANT polypeptide under conditions and for a time sufficient to permit binding of the agent to the ANT polypeptide and detecting such binding. In various preferred embodiments the host cell may be a prokaryotic cell or a eukaryotic cell. In certain other preferred embodiments the host cell may lack at least one isoform of an endogenous ANT, for example, due to a mutation in one or more endogenous ANT encoding genes. In certain other embodiments host cell expression of at least one gene encoding an endogenous ANT isoform is substantially impaired, for example, through the use of ANT nucleic acid-specific ribozyme or antisense constructs as provided herein, or through the use of ρ^0 cells, as also provided herein. According to other embodiments of this aspect of the invention, it may be preferred to use intact cells or, alternatively, to use permeabilized cells. Those having ordinary skill in the art are familiar with methods for permeabilizing cells, for example by way of illustration and not limitation, through the use of surfactants, detergents, phospholipids, phospholipid binding proteins, enzymes, viral membrane fusion proteins and the like; through the use of osmotically active agents; by using chemical crosslinking agents; by physicochemical methods including electroporation and the like, or by other permeabilizing methodologies.

In other aspects, the invention provides a method for identifying an agent that binds to an ANT polypeptide comprising contacting a candidate agent with a biological sample containing at least one recombinant ANT polypeptide under conditions and for a time sufficient to permit binding of the agent to the ANT polypeptide, and detecting such binding. The use of human ANT1, ANT2 and ANT3 according to these methods represent particularly preferred embodiments. Other preferred embodiments include the use of any ANT polypeptide or ANT fusion protein as provided herein. In some preferred embodiments an ANT fusion protein is used that is a GST fusion protein, and in other preferred embodiments the ANT fusion protein is a His-tagged fusion protein. As provided herein, the biological sample may be a cell, a mitochondrion, submitochondrial particles, a cell membrane (including any cellular

membrane as described herein), a cell extract, cell conditioned medium, a recombinant viral particle, a tissue homogenate or an isolated ANT. Detection of binding may be by any of a variety of methods and will depend on the nature of the candidate agent being screened. For example, certain candidate agents are inherently detectable as a consequence of their physicochemical properties, such as will be apparent to those skilled in the art and including spectrophotometric, colorimetric, fluorimetric, solubility, hydrophobic, hydrophilic, electrostatic charge, molecular mass or other physicochemical properties. As another example, certain candidate agents may be radioactively labeled with a readily detectable radionuclide, as is well known in the art.

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10 Certain candidate agents may also be directly or indirectly detectable by ANT protein affinity methodologies, for example by their ability to interfere with binding of an ANT-specific antibody to an ANT; or by their being removable from an assay solution using a protein affinity reagent that binds to a fusion polypeptide present as a portion of an ANT fusion protein. A candidate agent bound to an ANT polypeptide may be detected

15 by any method known for the detection, identification or characterization of relevant molecules, including spectrophotometric, mass spectrometric, chromatographic, electrophoretic, calorimetric or any other suitable analytical technique.

In another aspect the invention provides a method for identifying an agent that interacts with an ANT polypeptide comprising contacting a biological sample containing recombinant ANT with a detectable ANT ligand (or a known detectable molecule capable of binding to ANT) in the presence of a candidate agent, and comparing binding of the detectable ANT ligand (or known detectable ANT binding molecule) to recombinant ANT in the absence of the agent to binding of the detectable ANT ligand (or known detectable ANT binding molecule) to recombinant ANT in the presence of the agent, and therefrom identifying an agent that interacts with an ANT polypeptide. It will be appreciated that in certain preferred embodiments this aspect provides competitive binding assays wherein novel ANT ligands as provided hereinabove are useful. However, this aspect of the invention need not be so limited and may be modified to employ known detectable ANT binding molecules, in which

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30 case it should be pointed out that the selection of biological sample and/or of

recombinant ANT as provided by the present invention offer unexpected advantages heretofore unknown in the art. Examples of known detectable ANT-binding molecules include suitably labeled ATP, ADP, ATR, CATR, palmitoyl-CoA, bongkreikic acid, thyroxin, eosin Y and erythrosin B or other ANT-binding molecules known in the art.

5 (See, *e.g.*, Block et al., 1986 *Methods. Enzymol.* 125:658.) The use of human ANT1, ANT2 and ANT3 according to these methods represent particularly preferred embodiments. Other preferred embodiments include the use of any ANT polypeptide or ANT fusion protein as provided herein. In some preferred embodiments an ANT fusion protein is used that is a GST fusion protein, and in other preferred embodiments the

10 ANT fusion protein is a His-tagged fusion protein. As provided herein, the biological sample may be a cell, a mitochondrion, submitochondrial particles, a cell membrane (including any cellular membrane as described herein), a cell extract, cell conditioned medium, a recombinant viral particle, a tissue homogenate or an isolated ANT.

The ANT ligands compounds are preferably part of a pharmaceutical

15 composition when used in the methods of the present invention. The pharmaceutical composition will include at least one of a pharmaceutically acceptable carrier, diluent or excipient, in addition to one or more ANT ligands and, optionally, other components.

“Pharmaceutically acceptable carriers” for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remingtons

20 Pharmaceutical Sciences, Mack Publishing Co. (A.R. Gennaro edit. 1985). For example, sterile saline and phosphate-buffered saline at physiological pH may be used. Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. For example, sodium benzoate, sorbic acid and esters of *p*-hydroxybenzoic acid may be added as preservatives. Id. at 1449. In addition,

25 antioxidants and suspending agents may be used. Id.

“Pharmaceutically acceptable salt” refers to salts of the compounds of the present invention derived from the combination of such compounds and an organic or inorganic acid (acid addition salts) or an organic or inorganic base (base addition salts). The compounds of the present invention may be used in either the free base or

salt forms, with both forms being considered as being within the scope of the present invention.

The pharmaceutical compositions that contain one or more ANT substrates/ ligands compounds may be in any form which allows for the composition to be administered to a patient. For example, the composition may be in the form of a solid, liquid or gas (aerosol). Typical routes of administration include, without limitation, oral, topical, parenteral (*e.g.*, sublingually or buccally), sublingual, rectal, vaginal, and intranasal. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intrasternal, intracavernous, intrameatal, intraurethral injection or infusion techniques. The pharmaceutical composition is formulated so as to allow the active ingredients contained therein to be bioavailable upon administration of the composition to a patient. Compositions that will be administered to a patient take the form of one or more dosage units, where for example, a tablet may be a single dosage unit, and a container of one or more compounds of the invention in aerosol form may hold a plurality of dosage units.

For oral administration, an excipient and/or binder may be present. Examples are sucrose, kaolin, glycerin, starch dextrins, sodium alginate, carboxymethylcellulose and ethyl cellulose. Coloring and/or flavoring agents may be present. A coating shell may be employed.

The composition may be in the form of a liquid, *e.g.*, an elixir, syrup, solution, emulsion or suspension. The liquid may be for oral administration or for delivery by injection, as two examples. When intended for oral administration, preferred composition contain, in addition to one or more ANT substrates/ ligands compounds, one or more of a sweetening agent, preservatives, dye/colorant and flavor enhancer. In a composition intended to be administered by injection, one or more of a surfactant, preservative, wetting agent, dispersing agent, suspending agent, buffer, stabilizer and isotonic agent may be included.

A liquid pharmaceutical composition as used herein, whether in the form of a solution, suspension or other like form, may include one or more of the following adjuvants: sterile diluents such as water for injection, saline solution, preferably

physiological saline, Ringer's solution, isotonic sodium chloride, fixed oils such as synthetic mono or diglycerides which may serve as the solvent or suspending medium, polyethylene glycols, glycerin, propylene glycol or other solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. Physiological saline is a preferred adjuvant. An injectable pharmaceutical composition is preferably sterile.

10 A liquid composition intended for either parenteral or oral administration should contain an amount of ANT substrates/ ligands compound such that a suitable dosage will be obtained. Typically, this amount is at least 0.01 wt% of an ANT substrates/ ligands compound in the composition. When intended for oral administration, this amount may be varied to be between 0.1 and about 70% of the weight of the composition. Preferred oral compositions contain between about 4% and about 50% of ANT substrates/ ligands compound(s). Preferred compositions and preparations are prepared so that a parenteral dosage unit contains between 0.01 to 1% by weight of active compound.

20 The pharmaceutical composition may be intended for topical administration, in which case the carrier may suitably comprise a solution, emulsion, ointment or gel base. The base, for example, may comprise one or more of the following: petrolatum, lanolin, polyethylene glycols, beeswax, mineral oil, diluents such as water and alcohol, and emulsifiers and stabilizers. Thickening agents may be present in a pharmaceutical composition for topical administration. If intended for transdermal administration, the composition may include a transdermal patch or iontophoresis device. Topical formulations may contain a concentration of the ANT substrates/ ligands compound of from about 0.1 to about 10% w/v (weight per unit volume).

30 The composition may be intended for rectal administration, in the form, *e.g.*, of a suppository which will melt in the rectum and release the drug. The

composition for rectal administration may contain an oleaginous base as a suitable nonirritating excipient. Such bases include, without limitation, lanolin, cocoa butter and polyethylene glycol.

In the methods of the invention, the ANT substrates/ ligands compound(s) may be administered through use of insert(s), bead(s), timed-release formulation(s), patch(es) or fast-release formulation(s).

It will be evident to those of ordinary skill in the art that the optimal dosage of the ANT substrates/ ligands compound(s) may depend on the weight and physical condition of the patient; on the severity and longevity of the physical condition being treated; on the particular form of the active ingredient, the manner of administration and the composition employed. It is to be understood that use of an ANT substrates/ ligands compound in a chemotherapy can involve such a compound being bound to an agent, for example, a monoclonal or polyclonal antibody, a protein or a liposome, which assist the delivery of said compound.

15 EXAMPLES

The following Examples are offered by way of illustration and not by way of limitation.

EXAMPLE 1

CLONING AND EXPRESSION OF HIS-TAGGED HUMAN ANT

20 PROTEINS IN BACTERIA

A. PCR Amplification of ANT cDNAs

Total cellular RNA prepared from whole human brain was obtained from a commercial source (Clontech, Palo Alto, CA). The RNA was purified by treatment with RNase-free DNase I (Roche Molecular Biochemicals, formerly Boehringer Mannheim Biochemicals, Indianapolis, IN) using 1 ul of DNase I (10 u/ul) in a buffer containing 40 mM Tris-HCl, pH 7.0, 6 mM magnesium chloride and 2 mM calcium chloride for 30 minutes at 37°C. This treatment was followed by two phenol/chloroform extractions, one chloroform extraction and an ethanol precipitation in the presence of sodium acetate. The RNA pellet was collected by centrifugation,

washed with 70% ethanol, air dried, and resuspended in RNase-free sterile water. The RNA was reverse transcribed to generate cDNA using RNase H-deficient Reverse Transcriptase (SUPERScript™; Life Technologies, Rockville, MD).

ANT cDNAs were amplified by polymerase chain reactions (PCR) in a thermal cycler using the following primers, AMPLITAQ™ DNA Polymerase (Perkin-Elmer), and reagents and buffers supplied in a GENEAMP™ PCR Reagent Kit (Perkin-Elmer), according to the manufacturer's instructions. In the following representations of the PCR primers, underlined nucleotides indicate sequences complementary to the 5'-ends and 3'-ends of the ANT cDNAs and double-underlined nucleotides indicate recognition sequences for the restriction enzymes *XhoI* (recognition sequence: 5'-CTCGAG) and *Asp718* (recognition sequence: 5'-GGTACC).

For human ANT1 (huANT1; SEQ ID NO:1), the following primers were used:

Forward (sense):

15 5'-TTATATCTCGAGTATGGGTGATCACGCTTGGAGCTTCCTAAAG SEQ ID NO:4

and Reverse (antisense):

5'-TATATAGGTACCTTAGACATATTTTTTGATCTCATCATAACAAC SEQ ID NO:5.

20 For human ANT2 (huANT2; SEQ ID NO:2), the following primers were used:

Forward (sense):

5'-TTATATCTCGAGTATGACAGATGCCGCTGTGTCCTTCGCCAAG SEQ ID NO:6

and Reverse (antisense):

25 5'-TATATAGGTACCTTATGTGTACTTCTTGATTTCATCATAACAAG SEQ ID NO:7.

For human ANT3 (huANT3; SEQ ID NO:3), the following primers were used:

Forward (sense):

30 5'-TTATATCTCGAGTATGACGGAACAGGCCATCTCCTTCGCCAAA SEQ ID NO:8

and Reverse (antisense):

5'-TATATAGGTACCTTAGATCACCTTCTTGAGCTCGTCGTACAGG SEQ ID
NO:9.

B. Generation of ANT ExpressiJon Constructs

PCR products were digested with the restriction endonucleases *Xho*I and
5 *Asp*718 (both enzymes from Roche Molecular Biochemicals) according to the
manufacturer's recommendations using manufacturer-supplied reaction buffers.
Restricted DNAs were purified by horizontal agarose gel electrophoresis and band
extraction using the UltraClean GelSpin kit (Mo Bio Laboratories, Inc., Solana Beach,
CA).

10 The expression vector pBAD/His ("B" derivative; Invitrogen, Carlsbad,
CA) was used. This vector contains the following elements operably linked in a 5' to 3'
orientation: the inducible, but tightly regulatable, *araBAD* promoter; optimized *E. coli*
translation initiation signals; an amino terminal polyhistidine(6xHis)-encoding sequence
(also referred to as a "His-Tag"); an XPRESS™ epitope-encoding sequence; an
15 enterokinase cleavage site which can be used to remove the preceding N-terminal amino
acids following protein purification, if so desired; a multiple cloning site; and an in-
frame termination codon.

Plasmid pBAD/His DNA was prepared by digestion with the restriction
endonucleases *Xho*I and *Asp*718 according to the manufacturer's instructions and
20 subjected to horizontal agarose gel electrophoresis and band extraction using the
UltraClean GelSpin kit (Mo Bio Laboratories). Restricted ANT cDNAs were ligated
into the linearized plasmid with restricted expression vector DNA using T4 DNA ligase
(New England Biolabs, Beverly, MA) using the manufacturer's reaction buffer and
following the manufacturer's instructions. Competent *recA1 hsdR endA1E. coli* cells
25 (strain TOP10F'; Invitrogen, Catalog #C3030-03) were transformed with ligation
mixtures containing the prokaryotic vector construct according to the manufacturer's
instructions. Single colonies were selected and grown in 3-5 ml of LB broth
(Sambrook, J., Fritsch, E.F., and Maniatis, T., *Molecular Cloning: A Laboratory
Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989)

containing 50 µg/ml ampicillin (Roche Molecular Biochemicals). Plasmid DNA was isolated from the bacterial cultures using the WIZARDTM Plus Series 9600 Miniprep Reagents System (Promega, Madison, WI).

The recombinant huANT nucleotide sequences present in the expression constructs were determined and their authenticity confirmed relative to the published ANT sequences (Figure 1; *See Neckelmann et al., Proc. Nat'l. Acad. Sci. U.S.A.* 84:7580-7584 (1987) for huANT1; Battini et al., *J. Biol. Chem.* 262:4355-4359 (1987) for huANT2, and Cozens et al., *J. Mol. Biol.* 206:261-280 (1989) for huANT3.) by DNA sequencing using the PRISMTM Ready BIG DYETM Terminator Cycle Sequencing Kit (The Perkin-Elmer Corp., Norwalk, CT) and the following sequencing primers 5'-TATGCCATAGCATTTTATCC (SEQ ID NO:10) and 5'-CGCCAAAACAGCCAAGCT (SEQ ID NO:11). For each human ANT sequence, both primers are located inside the vector sequence adjacent to the DNA insertion. Sequence data was analyzed using the SEQUENCE NAVIGATORTM analysis software package (Perkin-Elmer). This huANT3 expression construct was named pMK3A-huANT3.

The expression plasmids encoding His-tagged human ANT1, ANT2 and ANT3 are referred to herein as follows: For human ANT1, "pMK1 (His-tagged huANT1)" or "pMK1"; for human ANT2, "pMK2 (His-tagged huANT2)" or "pMK2"; for human ANT3 "pMK3A (His-tagged hu ANT3)" or "pMK3A"; for human ANT3 from which extraneous linker N-terminal amino acids are deleted as detailed below, "pMK3B (His-tagged hu ANT3, shortened epitope linker)" or "pMK3B". Plasmids pMK1, pMK2 and pMK3A have been deposited at the American Type Culture Collection (ATCC; Manassas, VA) on November 3, 1998, and given the accession numbers ATCC _____, ATCC _____ and ATCC _____, respectively.

The expression constructs comprising nucleotide sequences encoding human ANT1 (pMK1-huANT1) and human ANT2 (pMK2-huANT2) were restriction mapped to confirm their structures. The nucleotide sequences of plasmids pMK1-huANT1 and pMK2-huANT2 are determined using the methods and primers (SEQ ID NOS:10 and 11) described above.

Treatment of the recombinant huANT3 protein expressed from pMK3A-huANT3 with enterokinase liberates the His-Tag/XPRESSTM epitope polypeptide from the huANT3 protein; however, the resultant huANT3 protein comprises several extraneous N-terminal amino acids (*i.e.*, Pro-Ser-Ser-Ser-Met, where “Met” indicates the amino acid encoded by the translation initiation codon of huANT3). Although the extraneous amino acids probably have little or no effect on the recombinant huANT3 protein, a derivative expression construct in which the nucleotide sequence encoding the extraneous amino acids are deleted was prepared in the following manner.

The QUIK-CHANGETM Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) essentially according to the manufacturer’s instructions. Briefly, a reaction mixture comprising purified pMK3A-huANT3 DNA, the mutagenic oligonucleotide primers 5’-GGAGATGGCCTGTTCCGTCATCTTATCGTCATCGTCGTACAGATC (SEQ ID NO:12; the underlined sequence is the reverse complement of the 5’ end of the huANT3 reading frame), and 5’-
 15 GATCTGTACGACGATGACGATAAGATGACGGAACAGGCCATCTCC (SEQ ID NO:13; the underlined sequence corresponds to the 5’ end of the huANT3 reading frame), *Pfu* DNA polymerase and dNTPs in manufacturer-supplied reaction buffer was prepared. The mutagenic oligonucleotide primers were present in excess and cycles of DNA synthesis was carried out in a thermal cycler according to the manufacturer’s
 20 protocol. The reaction products were treated with the restriction enzyme *DpnI*, which cleaves methylated and hemi-methylated DNAs but leaves unmethylated DNA (*i.e.*, annealed products of the reaction) intact, and used to transform EPICUREAN COLITM XL-1-Blue *E. coli* cells (Stratagene). Plasmid DNA was prepared from twelve randomly selected transformants and the nucleotide sequence of the region containing
 25 the multiple cloning site cassette was determined according to the methods described above. Of the twelve plasmids, only one retained the original sequence found in pMK-huANT3, and three contained undesired point mutations. One of the eight “correct” plasmids was chosen and named pMK3B-huANT3.

C. Expression of His-Tagged huANT3

Cultures of *E. coli* cells containing pMK3A-huANT3 were grown in LB media containing 50 ug/ml ampicillin to mid-log phase ($OD_{600} \sim 0.5$) and induced for 3-4 hours with increasing doses of arabinose (i.e., 0.00002%, 0.0002%, 0.002%, 0.02%, and 0.2 %). One ml of each culture was centrifuged at 5,000 x g for 10 minutes at 4°C to pellet the cells. Cell pellets were resuspended, and the cells were lysed, by adding 100 ul of Phosphate Buffered Saline (PBS; pH 7.4) containing 1% cholate, 1% n-dodecyl maltoside, and 0.1% 2-mercaptoethanol (in the preceding text, and throughout the specification, unless specified otherwise, all chemicals are from Sigma, St. Louis, MO). Total protein content in the lysates was determined using the BCA (bicinchoninic acid; Smith *et al.*, 1985, *Anal. Biochem.* 150:76-85) Protein Assay kit (Pierce Chemical Co., Rockford, IL). Ten µg of total protein were loaded per lane onto an SDS polyacrylamide gel, electrophoresed and transferred to a nitrocellulose membrane (HYBOND™ ECL Nitrocellulose Membrane, Amersham Pharmacia Biotech, formerly Amersham Life Sciences, Piscataway, NJ). Human ANT3 fusion proteins were detected in a western blot using ANTI-XPRESS™ Antibody (Invitrogen) and horseradish peroxidase-conjugated anti-mouse secondary antibody (Amersham Pharmacia Biotech) according to the manufacturers' instructions.

The results are shown in Figure 3. From left to right in the figure, the following samples are shown: lanes "M", molecular weight markers; lane "0", untransformed *E. coli* cells; lane "o/n", *E. coli* comprising pMK3A-huANT3 grown overnight without induction; lane "1" – "5", *E. coli* comprising pMK3A-huANT3 grown induced with increasing doses of arabinose (0.00002%, 0.0002%, 0.002%, 0.02% and 0.2 %, respectively). As expected, untransformed (lane 0) and uninduced (lane o/n) *E. coli* showed no XPRESS™-huANT3 material. However, expression of recombinant ANT3 fusion protein with a molecular weight of 36.6 kD was observed in lanes 3 and 4 (0.002% and 0.02% arabinose, respectively). No XPRESS™-huANT3 material was detected in lanes 1 and 2 (0.00002% and 0.0002% arabinose, respectively) indicating that the degree of induction was insufficient under these conditions.

Cells that were grown in the presence of the highest concentration of arabinose (0.2%, lane 5) began to lyse and died before the time of harvest; consequently, no recombinant protein was detected. This indicated that very high expression of recombinant huANT in *E. coli* caused cell death, as is sometimes the case during overexpression of heterologous proteins in bacteria.

D. Recombinant huANT3 Localizes to the Bacterial Membrane

In order to locate the expressed human ANT 3 within *E. coli* cells, cells were grown in culture and induced with arabinose as described above, and then fractionated into different compartments (e.g., membranes, inclusion bodies and cytosol). Bacteria were pelleted by centrifugation at 5,000 x g for 10 minutes at 4°C. The cell pellets were resuspended in 1/10 volume of cell buffer A (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 100 ug/ml lysozyme, and 0.1% Triton X-100) and incubated for 15 minutes at 30°C in an orbital shaker. The cell mixture was sonicated for 2 minutes and membranes were pelleted by centrifugation at 12,000 x g for 15 minutes at 4°C. The supernatant, representing the cytosol, was removed for analysis (Figure 4, lane 4), as was a portion of the pellet containing membranes and inclusion bodies (Figure 4, lane 3). The remaining portion of the pellet was washed twice with cell buffer B (10 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, and 1 mM DTT) and centrifuged at 12,000 x g for 15 minutes at 4°C. The pellet was resuspended in cell buffer C (20 mM Tris-HCl, pH 8.0, 100 mM sodium chloride, and 6 M guanidinium hydrochloride) and incubated for 1 hour at room temperature. The solution was then centrifuged at 12,000 x g for 15 minutes at 4°C. The supernatant (containing solubilized inclusion bodies; lane 1, Figure 4) and the pellet (containing insoluble inclusion bodies; lane 2, Figure 4) were analyzed by Western blotting as described above.

The results are shown in Figure 4. Recombinant huANT 3 (molecular weight 36.6 kD) was detected in lanes 2, 3, and 4, as well as the positive control lane (+) (total cell lysate previously tested for presence of ANT3 protein by Western immunoblot analysis, as described above). The greatest amount of recombinant huATN3 was detected in lane 3, which represents the membrane fraction. This

indicates that the majority of the huANT3 fusion protein integrated into the *E. coli* cellular membrane. Smaller protein signals were visible in lanes 2 and 4, representing the insoluble inclusion body fraction which might have contained some membranes with integrated ANT 3, and the cytosolic fraction where protein synthesis takes place.

- 5 No protein was detectable in the soluble inclusion body fraction in lane 1, indicating that controlled expression of ANT3 in the bacteria did not result in the formation of inclusion bodies, which is an undesirable consequence of over-expression of some heterologous proteins in bacteria.

E. Purification of ANT Proteins

- 10 ANT proteins, and ANT fusion proteins, produced by the expression systems described herein have been purified using a variety of methods. The purification of ANT proteins, particularly human ANT proteins, is described in this Example.

- 15 Regardless of which of following protein purification methods is used, or others that can be derived from the present disclosure, it is important to add sufficient amounts of DNase and RNase to eliminate the viscosity associated with some bacterial lysates (typically 10 µg/mL of each enzyme; both from Roche Biochemicals) when the bacterial cells are lysed (or immediately thereafter). An alternative or additional means by which viscosity has been minimized and ANT solubility has been optimized is
- 20 vigorous sonication, as opposed to standard sonication, of the lysates. The term “vigorous sonication” refers to, for example, sonication with a Branson Sonifier (Model 450) 2x (30 seconds each time) at 50% duty cycle and 80% output using a tapered, flat-tipped probe (as opposed to sonication with a cup and horn apparatus). Although either type of sonication will suffice, better yields have typically been observed when vigorous
- 25 sonication has been used.

Furthermore, in various ANT purification methods that have been used, it was often desirable to make the lysate at least 1% Triton-X, in order to solubilize the maximum possible amount of ANT protein, after which insoluble material is removed by a high-speed (i.e., about 100,000 g) spin. Typically, protease inhibitors such as, for

example, pepstatin, leupeptin, phenylmethylsulfonyl fluoride (PMSF) and/or aprotinin (all from Sigma) have been present at effective levels (typically 10 µg/mL) during the preparation. Depending on the particular ANT protein or ANT fusion protein being isolated, all four protease inhibitors or any effective combination thereof are used. For example, in preparations of GST-huANT3 fusion proteins, best results were seen when all four protease inhibitors were used, although acceptable results have been obtained when only leupeptin and pepstatin were used.

One method incorporates novel methods with several techniques previously used only for purifying ANT proteins from non-human mammals, i.e., bovine cardiac tissue and rats (Aquila *et al.*, 1982, *Hoppe-Seyler's Z. Physiol. Chem.* 363:345-349; and Sterling, 1986, *Endocrinology* 119:292-295). In brief, bacterial cells expressing a GST-ANT3 fusion protein were lysed by lysozyme treatment, and ¹⁴C-palmityl-CoA (Sigma) was added at a concentration of 50 nmol per gram of *E. coli*. Because it associates with ANT proteins, ¹⁴C-palmityl-CoA acts as a radiolabeled tracer that can be used to follow the ANT protein in subsequent purification steps. The lysates were then sonicated and made 6% Triton X-100 (Sigma) and incubated at 4°C for 1 hr to solubilize material. A high-speed spin was used to remove insoluble material, and the resulting solute was applied either (1) for small scale preparations, to hydroxyapatite beads (Bio-Rad Laboratories, Hercules, CA), or (2) in the case of larger preparations (i.e., ≥1 liter of bacterial culture), to a hydroxyapatite column (Bio-Rad) essentially according to the manufacturer's instructions. Unlike other intramembrane mitochondrial proteins, ANT has a low affinity for hydroxyapatite (Klingenberg *et al.*, 1978, *Biochim. et Biophys. Acta* 503:193-210). The hydroxyapatite column was eluted with Column Buffer A (10mM MOPS, pH 7.2, 100mM NaCl, 9.5% Triton X100) and washed with Column Buffer B (10 mM MOPS, pH 7.2, 100mM NaCl, 400mM sodium phosphate). Non-recombinant ANT proteins from non-human species are eluted in the void volume with Column Buffer A, and the GST-huANT3 fusion protein was expected to be present in the void volume as well; Column Buffer B was used to wash the column in the event that GST-huANT3 fusion protein behaves differently. Samples were collected in such a manner as to have a final concentration of 30 of mM octyl

glucoside (Calbiochem), a nonionic detergent that helps solubilize ANT proteins with minimal effect on activity (Sterling, 1986, *Endocrinol.* 119: 292-295). The bead-extracted supernatant or column eluent was collected, and Triton X-100 was removed therefrom using the EXTRACTI-GEL™ affinity matrix (Pierce) essentially according to the manufacturer's instructions (see also Berman *et al.*, 1985, *Biochemistry* 24:7140-7147).

Varying amounts of GST-huANT3 prepared in the above manner were subject to PAGE and the gel was stained using a colloidal blue protein stain (Novex, San Diego, CA). The stained gel displayed a single band having a molecular weight corresponding to that predicted for the fusion protein. Based on the intensity of bands from samples of varying volumes, and the known volume of the preparation and minimal sensitivity of the stain, the yield from 100 mL of bacterial culture was estimated to be about 50 ug. In one of the lanes of the gel, approximately 500 ng of protein was loaded, and no contaminating bands were detected; this indicates that the GST-huANT3 protein was from at least about 90% pure to at least about 95% pure.

GST-huANT3 fusion proteins (see preceding Examples) have been purified by this method, and other ANT fusion proteins, including His-tagged huANT3 and other His-tagged ANT proteins, are purified in like fashion. Purified huANT fusion proteins are used to produce purified human ANT proteins as follows.

GST-huANT fusion proteins are further purified via glutathione-agarose beads (Sigma) essentially according to the manufacturer's instructions. In brief, a solution comprising GST-huANT fusion proteins is contacted with glutathione-agarose beads, and the beads are washed to release undesirable contaminants. Next, the [bead:GST-huANT] complexes are treated with an appropriate enzyme, i.e., one that separates the huANT polypeptide from the remainder of the fusion protein. In the case of the GST-huANT3 fusion protein described herein (i.e., that encoded by pMK3C), thrombin (Sigma) cleaves the fusion protein in such a manner so as to produce two polypeptides: a first polypeptide corresponding to the GST moiety, and a second polypeptide which corresponds to human ANT3 with an additional six amino acids (i.e., Gly-Ser-Pro-Gly-Ile-Leu) present at its N-terminus.

His-tagged huANT fusion proteins are further purified via Nickel-coated resins (such as, *e.g.*, PROBOND™ Ni²⁺ charged agarose resin; Invitrogen) essentially according to the manufacturer's instructions. In brief, a solution comprising His-tagged huANT fusion proteins is contacted with the Nickel-coated resin, and the resin is washed to release undesirable contaminants. Next, the [resin:His-tagged huANT] complexes are treated with an appropriate enzyme, *i.e.*, one that separates the huANT polypeptide from the remainder of the fusion protein. In the case of the His-tagged huANT3 fusion proteins described herein, enterokinase (Sigma, or EKMAX™ from Invitrogen may be used) cleaves the fusion protein in such a manner so as to produce two polypeptides: a first polypeptide comprising the His-tag and XPRESS™ epitope moieties, and a second polypeptide which corresponds to human ANT3. If the expression construct used is pMK3A, the resultant purified human ANT3 protein has an additional four amino acids (*i.e.*, Pro-Ser-Ser-Ser) at its N-terminus. If pMK3B is the expression construct present in the cells from which His-tagged huANT3 is isolated, the resultant purified human ANT3 protein has the sequence of native huANT3, *i.e.*, SEQ ID NO:3.

In both of the preceding purification steps, an ANT fusion protein bound to a solid support is treated with an enzyme (*i.e.*, thrombin or enterokinase) that liberates an ANT protein from the remainder of the fusion protein, which remains bound to the solid support. ANT protein is released into the liquid phase which is then collected to generate a solution comprising the ANT protein and some amount of the liberating enzyme. The amount of liberating enzyme needed is minimal because the treatment is catalytic in nature; nevertheless, some enzyme remains in the preparation. If desired, enzyme molecules may be removed from the preparation using any of a variety of means known in the art. For example, an enzyme may be removed from a solution by contacting the solution with a resin conjugated to a ligand having a high affinity for the enzyme. In the case of enterokinases, one such resin is the EK-AWAY™ resin (Invitrogen) which comprises the soybean trypsin inhibitor having a high affinity for enterokinases. Methods of treating GST fusion proteins with thrombin and purifying the desired recombinant protein have been described previously (see, for

example, Smith and Corcoran, Unit 16.7 in Chapter 16 in *Short Protocols in Molecular Biology* 2nd Ed., Ausubel *et al.*, eds, John Wiley & Sons, New York, New York, 1992, pages 16-28 to 16-31. In general, however, any suitable means for separating the liberating enzyme from any given ANT protein may be used.

5 F. Growth Inhibition

As noted in the above discussion of the results presented in Figure 3, very high expression of recombinant huANT3 in *E. coli* caused cell death. Such a result is sometimes observed during over-expression of heterologous proteins in bacteria. Although not wishing to be bound by any particular theory, because the recombinant huANT3 protein localized to the bacterial membrane, and because ANT3 functions as an ATP/ADP exchanger in the inner mitochondrial membrane and under appropriate conditions may exhibit pore properties suggestive of a role in membrane permeability, one possible explanation for the observed cell death would be an inappropriate enhancement of the permeability of the bacterial membrane. If this in fact the case, inhibitors of mitochondrial ANT might prevent the death of *E. coli* overexpressing huANT3. As noted above, under certain conditions atractyloside or bongkreikic acid may exhibit inhibition of ANT activity, such that either of these inhibitors, other known ANT-active agents and potentially other ANT ligands as provided herein may be employed in the instant Example described using bongkreikic acid.

In order to test this hypothesis, the following experiments are carried out. *E. coli* harboring pMK3A-huANT3 are grown with no arabinose or with 0.2% or more arabinose, the latter concentration having been previously shown to induce toxic levels of huANT3, and various concentrations (0, 5, 20, 50 and 200 μ M) of bongkreikic acid (Biomol Research Laboratories, Inc., Plymouth Meeting, MA), an inhibitor of ANT (Henderson and Lardy, 1970, *J. Biol. Chem.* 245:1319-1326) that binds to ANT (see, e.g., Vignais *et al.*, 1976, *Biochim. Biophys.* 440:688-696). The ability of bongkreikic acid to prevent the lysis of *E. coli* overexpressing huANT3, or any other ANT protein for that matter, indicates that the toxic effect of such overexpression is due to an activity associated with normally functioning ANT.

ANT proteins produced by this expression system, and others described herein, are also purified using known methods for purifying ANT proteins from humans and other mammals. See for example, Klingenberg et al., 1978 *Biochim. Biophys. Acta* 503:193-210; Aquila et al., 1982 *Hoppe-Seyler's Z. Physiol. Chem.* 363:345-349; and
 5 Sterling, 1986 *Endocrinol.* 119:292-295.

The bacterial toxicity of extreme overexpression of ANT in this system can be used to screen and identify novel inhibitors of ANT, as such compounds will be expected to also prevent lysis of *E. coli* overexpressing ANT proteins. In order to achieve a greater degree of specificity for the ANT protein produced from an expression
 10 vector, the yeast expression system for ANT proteins (see Example 4, *infra*) is used in a mutant yeast strain that is resistant to bongkreikic acid (Lauquin *et al.*, 1975 *FEBS Letters* 35:198-200).

EXAMPLE 2:

EXPRESSION OF GST-HUANT3 FUSION PROTEINS

15 A. Generation of GST-huANT3 Expression Constructs

Human ANT3 cDNA was amplified from pMK3A-huANT3 by PCR as in Example 1 but using the following primers. In the following representations of PCR primers, underlined nucleotides indicate sequences complementary to the 5'-ends and 3'-ends of the ANT cDNAs and double-underlined nucleotides indicate recognition
 20 sequences for the restriction enzymes *XhoI* (recognition sequence: 5'-CTCGAG) or *EcoRI* (recognition sequence: 5'-GAATTC).

The primers used for PCR amplification were:

Forward (sense):

5'-CCCGGGGAATTCTGATGACGGAACAGGCCATCTCC SEQ ID NO:14

25 and Reverse (antisense):

5'-CCCGGGGCTCGAGTTAGAGTCACCTTCTTGAGCTC SEQ ID NO:15

The expression vector pGEX-4T-2 (Amersham Pharmacia Biotech) was used to generate huANT3 fusion proteins comprising an enzymatic polypeptide and an ANT polypeptide. This vector comprises a *lacI*^q (repressor) gene a *tac* promoter

operably linked to a glutathione S-transferase (GST) gene from *Schistosoma japonicum*. (Smith *et al.*, 1988, *Gene* 67:31-40), the coding sequence of which has been modified to comprise a thrombin cleavage site-encoding nucleotide sequence immediately 5' from a multiple cloning site. GST fusion proteins can be detected by Western blots with anti-
 5 GST or by using a colorimetric assay; the latter assay utilizes glutathione and 1-chloro-2-4-dinitrobenzene (CDNB) as substrates for GST and yields a yellow product detectable at 340 nm (Habig *et al.*, 1974, *J. Biol. Chem.* 249:7130-7139). GST fusion proteins produced from expression constructs derived from this expression vector can be purified by, e.g., glutathione affinity chromatography, and the desired polypeptide
 10 released from the fusion product by thrombin. Thus, this expression vector provides for the rapid purification of fusion proteins, and release of proteins with relatively few extraneous N-terminal amino acids, although the resulting recombinantly produced protein contains two additional amino acids at the amino terminus (Gly-Ser). The *tac* promoter may be induced by the addition to cultured cells of, e.g., 1-5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG; Fluka, Milwaukee, WI) and provides for high-
 15 level expression.

Plasmid pGEX-4T-2 was prepared by digestion with the restriction endonucleases *EcoRI* and *Asp718* according to the manufacturer's instructions and subjected to horizontal agarose gel electrophoresis and band extraction using the
 20 UltraClean GelSpin kit (Mo Bio Laboratories). Restricted ANT cDNAs were ligated with the restricted expression vector DNA as described in the preceding Example. Single colonies were selected for grown in 3-5 ml of LB broth containing 50 ug/ml ampicillin (Roche Molecular Biochemicals), and plasmid DNA was isolated from the bacterial cultures using the WIZARDTM *Plus* Series 9600 Miniprep Reagents System
 25 (Promega). To confirm their authenticity, the recombinant huANT nucleotide sequences present in the pGEX derivative plasmid were determined as described in the preceding Example using the previously described oligonucleotide primers and 5' and 3' PGEX Sequencing Primers (Amersham Pharmacia Biotech).

The resultant GST-huANT3 expression construct was named pMK3C-
 30 GST-huANT3. Thrombin treated recombinant huANT3 protein produced from the

pMK3C-GST-huANT3 expression construct actually includes a few extraneous N-terminal amino acids, i.e., Gly-Ser-Pro-Gly-Ile-Leu-Met, where “Met” indicates the amino acid encoded by the translation initiation codon of huANT3. There is, however, no evidence that the extraneous six amino terminal amino acids have any effect on the resultant recombinant huANT3 protein.

In order to confirm expression of the GST-huANT3 fusion protein, the following experiments were carried out. Eight independently isolated pMK3C-GST-huANT3 transformants and one control (vector-transformed) isolate were grown overnight in LB-ampicillin and then diluted 1:20 in 2 ml of fresh media. After 3 hours of growth at 37°C, IPTG was added to a final concentration of 0.1 mM. Cell growth was continued for 2 hours, after which 1.5 of cells were transferred to microfuge tubes, pelleted, resuspended in 300 uL of cold PBS containing 1% Triton X-100, and sonicated twice for 8 seconds. The sonicates were spun for 5 min. at 4°C, the supernatant was transferred to fresh microfuge tubes and 50 uL of glutathione-agarose beads (Sigma) were added to produce a 50% slurry. After a 5 min. incubation at ambient temperature, the beads were spun and washed with 1 ml of PBS three times. The washed pellet was resuspended in SDS spl buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5% beta-mercaptoethanol and sufficient bromophenol blue to provide visible coloration), and 30 uL of each preparation (equivalent to 15 uL of culture) was subjected to SDS-PAGE. The gel was stained using a Colloidal Coomassie (G-250) Staining Kit (Novex, San Diego, CA). A band of the predicted molecular weight of the GST-huANT3 fusion protein was readily apparent, with the same intensity, in each of the 8 preparations from pMK3C-GST-huANT3 transformants; this band was absent in the control preparation.

The expression plasmid encoding human ANT3-glutathione-S-transferase (GST) fusion protein is referred to as “pMK3C (GST-huANT3)” or “pMK3C”. Plasmid pMK3C has been deposited at the American Type Culture Collection (ATCC; Manassas, VA) on November 3, 1998, and given the accession number ATCC.

B. Western Blot Analysis of Expression of huANT3 Fusion Proteins

E. coli transformed with either (1) pMK3A-huANT3 (the pBAD/His-huANT3 expression construct) or (2) pMK3C-GST-huANT3 (the pGEX/GST-huANT3 expression construct) were lysed by the addition of lysozyme (100 µg/µl; Sigma) for 20 min at room temperature, followed by one freeze/thaw cycle. The negative control for the former transformant was a parallel culture of the transformed cells that had not undergone arabinose induction. The control for the latter transformant was a parallel culture of *E. coli* that had been transformed with the pGEX-4T-2 vector only.

Total protein concentrations of each lysate were determined using the BCA Protein Assay kit (Pierce Chemical Co.), and equivalent amounts of total protein from each lysate preparation were mixed with equivalent volumes of 2x Laemmli electrophoresis buffer and subjected to SDS-PAGE. The proteins were electrophoretically transferred to nitrocellulose, which was then contacted with antibodies against the appropriate epitope included in each vector (i.e., ANTI-XPRESSTM from Invitrogen for pMK3A-huANT3 and polyclonal goat anti-GST from Amersham Pharmacia Biotech, formerly Nycomed Amersham plc and Pharmacia & UpJohn Inc. for pMK3C-GST-huANT3).

In a separate experiment, the bacterial lysate from the pMK3C-GST-huANT3 transformants was incubated with agarose-glutathione beads (Sigma) according to the manufacturer's instructions (see the preceding section and Smith *et al.*, Expression and Purification of Glutathione S-Transferase Fusion Proteins, Unit 16.7 of Chapter 16 in: *Short Protocols in Molecular Biology*, 2nd Ed., Asubel *et al.*, eds., John Wiley & Sons, New York, New York, 1992, pages 16-28 to 16-31). The beads were suspended in Laemmli sample buffer and subjected to SDS-PAGE and Western analysis as described above. Although the yield of GST-huANT3 was low, perhaps because the fusion protein is inserted into the bacterial membrane, a sufficient amount of material was recovered for the experiment.

The results (Figure 5) show that a specific band of the predicted molecular weight (His-Tag + enterokinase site + antigenic site + huANT3 = 38 kDa) was observed in the arabinose induced *E. coli* that were transformed with the pBAD/his-

huANT3 vector, but was absent in the non-induced control culture. Similarly, a band corresponding to GST-huANT3 was observed in the pMK3C-GST-huANT3-transformed *E.coli*, while only the unaltered GST band was observed in control *E.coli* transformed with the expression vector. Purification of the GST-huANT3 fusion protein using agarose-GSH beads produced a band of equivalent size to that observed in the crude lysate of pMK3C-GST-huANT-transformed bacteria.

EXAMPLE 3 EXPRESSION OF ANT3 IN INSECT CELLS

10 A. Generation of Baculovirus Expression Constructs

DNA comprising nucleotide sequences encoding huANT3 was amplified by PCR from a whole human brain cDNA library (Clontech) using the following primers. In the following representations of PCR primers, underlined nucleotides indicate sequences complementary to the 5'-ends and 3'-ends of the ANT cDNAs and double-underlined nucleotides indicate recognition sequences for the restriction enzymes *Bam*HI (recognition sequence: 5'-GGATCC) or *Eco*RI (recognition sequence: 5'-GAATTC).

The PCR primers used were:

Forward (sense):

20 5'-TTATAGGGATCCATGACGGAACAGGCCATCTCCTTCGCCAAA SEQ ID
NO:16

and Reverse (antisense):

5'-TTAAAGAGAATTCTTAGATCACCTTCTTGAGCTCGTCGTACAG SEQ ID
NO:17.

25 PCR products were digested with the restriction endonucleases *Bam*HI (New England Biolabs) and *Eco*RI (New England Biolabs) according to the manufacturer's recommendations. Subsequent purification was carried out by horizontal agarose gel electrophoresis and band extraction using the UltraClean GelSpin kit (Mo Bio Laboratories, Inc.).

The Baculovirus transfer vector pBlueBacHis2 (B version, Invitrogen) comprises, in 5' to 3' orientation, a constitutive polyhedrin promoter operably linked to nucleotide sequences encoding (1) a translation initiation sequence, (2) an N-terminal polyhistidine sequence, (3) an XPRESSTM epitope tag for detection and purification of the recombinant protein and (4) an enterokinas cleavage site, followed by a multiple cloning site wherein cDNAs can be inserted.

The transfer vector pBlueBacHis2 was prepared by digestion with the restriction endonucleases *Bam*HI and *Eco*RI according to the manufacturer's recommendation, and the restricted DNA was subject to horizontal agarose gel electrophoresis and band extraction using the UltraClean GelSpin kit (Mo Bio Laboratories, Inc.). The restricted PCR products were ligated with the restricted expression vector DNA as in the preceding Examples.

Competent *E. coli* TOP10F' cells (Invitrogen) were transformed with the ligation reaction following the manufacturer's instructions. Single colonies were selected for growth in 3-5 ml of LB broth containing 50 ug/ml ampicillin. Plasmid DNA was isolated from the bacterial cultures using the WIZARDTM Plus Series 9600 Miniprep Reagents System (Promega).

The recombinant ANT gene sequences were determined and their authenticities confirmed (SEQ ID NOS:1, 2 and 3 correspond to human ANT1s 1, 2 and 3, respectively) by DNA sequencing using the Prism Ready Dye Terminator Cycle Sequencing Kit (Perkin-Elmer, Catalog #402080) and the following primers: Polyhedrin Forward Sequencing Priming Site, 5'- AAATGATAACCATCTCGC (SEQ ID NO:18); Baculovirus Reverse Sequencing Priming Site, 5'- ACTTCAAGGAGAATTTCC (SEQ ID NO:19); primers internal to the ANT 3 coding sequence (sense strand), 5'-ACTTCGCCTTCACGGATA (SEQ ID NO:20); and 5'- TACGGCCAAGGGCATTCT (SEQ ID NO:21); primers internal to the ANT 3 coding sequence (antisense strand), 5'- TGAAGCGGAAGTTCCTAT (SEQ ID NO:22); and 5'- ATGCCGGTTCCCGTACGA (SEQ ID NO:23). Sequence data were analysed using the SEQUENCE NAVIGATORTM analysis software package (Perkin-Elmer). An isolated plasmid having the correct sequence was named pMK4A-huANT3.

Although pMK4A-huANT3 contains authentic huANT3-encoding sequences, the ANT3 reading frame is not synchronous with the reading frame of the His-Tag/XPRESSTM epitope of the expression vector. Accordingly, pMK4A-huANT3 is not expected to produce recombinant ANT protein, although cells harboring it may be used as controls.

In order to generate an in-frame derivative of pMK4A-huANT3, the plasmid was mutagenized using the QUIK-CHANGETM Site-Directed Mutagenesis Kit (Stratagene) as in Example 1, except that the mutagenic oligonucleotide primers used were 5'-GGCCTGTTCCGTCATCTTATCGTCATCGTCG (SEQ ID NO:24; the underlined sequence is the reverse complement of the 5' end of the huANT3 reading frame), and 5'-CGACGATGACGATAAGATGACGGAACAGGCC (SEQ ID NO:25; the underlined sequence corresponds to the 5' end of the huANT3 reading frame). Several transformants were isolated, and plasmid DNA purified therefrom. The nucleotide sequences of the plasmid DNAs were determined and one having the "correct" sequence was identified and named pMK4B-huANT3.

The baculovirus expression plasmids encoding human ANT3 are referred to as "pMK4A (baculovirus shuttle, out-of-frame hu ANT3) or "pMK4A"; and "pMK4B (baculovirus shuttle, in-frame hu ANT3)" or "pMK4B". Plasmid pM4B has been deposited at the American Type Culture Collection (ATCC; Manassas, VA) on November 3, 1998, and given the accession number ATCC.

In order to insert sequences encoding the huANT3 protein (and associated regulatory sequences) into the baculovirus genome, insect (*Spodoptera frugiperda*, Sf9) cells (MAXBACTM, Invitrogen) were co-transfected with the baculoviral transfer construct pMK4B-huANT3 and linear baculoviral (*Autographa californica* nuclear polyhedrosis virus, AcMNPV) DNA engineered to contain a promoterless 3' fragment of the *lacZ* gene (BAC-N-BLUETM, Invitrogen) using the BAC-N-BLUETM Transfection Kit (Invitrogen) following the manufacturer's instructions. Recombinant baculovirus plaques express functional beta-galactosidase and were identified as blue plaques in the presence of X-gal (5-bromo-4-chloro-3-indoyl-beta-D-galactosidase). These recombinant viruses are expression constructs that

express human ANT3 polypeptide in insect cells, as shown by the following experiments.

B. Western Blot Analysis of Baculovirus Expression Systems

High titer viral stock was produced, and recombinant protein was expressed in infected Sf9 cells according to the manufacturer's instructions (see also Piwnica-Worms, Expression of Proteins in Insect Cells Using Baculovirus Vectors, Section II of Chapter 16 in: *Short Protocols in Molecular Biology*, 2nd Ed., Asubel *et al.*, eds., John Wiley & Sons, New York, New York, 1992, pages 16-32 to 16-48; Kitts, Chapter 7 in: *Baculovirus Expression Protocols*, Methods in Molecular Biology, Vol. 39, C.R. Richardson, Ed., Humana Press, Totawa, NJ, 1995, pages 129-142).

Transfected cells were pelleted by centrifugation and lysed by adding 100 ul of MSB buffer (210 mM mannitol (Sigma), 70 mM sucrose (Fluka), 50 mM Tris-HCl, pH 7.4, 10 mM EDTA) and performing three freeze-thaw cycles. A total cellular fraction, a cytosolic fraction, a submitochondrial partical fraction, a mitochondrial fraction and a plasma membrane fraction were prepared as follows. The cell lysate was centrifuged at 600 g for 10 minutes at 4°C to prepare a plasma membrane pellet. The supernatant was removed and set aside. The plasma membrane pellet was washed with 100 ul of MSB, centrifuged at 600 g for 10 minutes at 4°C, and used for the analysis. The supernatant was removed, combined with the first supernatant and mixed. Half of the supernatant was used to prepare a mitochondrial fraction and a cytosolic fraction by centrifugation at 14,000 g for 15 minutes at 4°C; the pellet represents the mitochondrial fraction and the supernatant represents the cytosol. The other half of the supernatant was centrifuged at 14,000 g for 15 minutes at 4°C to produce a mitochondria-containing pellet that was resuspended in MSB, incubated with 0.25 mg/ml digitonin (Roche Molecular Biochemicals, formerly Boehringer Mannheim, Indianapolis, IN) for 2 min and sonicated for 3 min at 50% duty cycle in a cup-horn sonicator to produce submitochondrial particles (SMPs).

The protein content for each fraction was determined using the BCA Protein Assay kit (Pierce Chemical Co.), and 8 ug of total protein were loaded per lane

onto an SDS polyacrylamide gel, electrophoresed and transferred to a HYBOND™ ECL Nitrocellulose Membrane (Amersham Life Science). Fusion proteins were detected in a western blot using ANTI-XPRESS™ Antibody (Invitrogen, Catalog #R910-25) and horseradish peroxidase-conjugated anti-mouse secondary antibody (Amersham Life Science) following the manufacturers' instructions.

The results of the Western analysis are shown in Figure 6. Recombinant GST-huANT3 fusion protein (molecular weight 36.6 kD) was detected in total cells, mitochondria, submitochondrial particles and the plasma membrane. The signal was most intense in mitochondria and submitochondrial particles, whereas no band was detectable in the cytosolic fraction. These data suggest that the human recombinant huANT3 fusion protein integrated into the mitochondrial membranes much more efficiently than into the plasma membranes. Furthermore, all of the recombinant protein integrated into membranes since no signal was detected in the cytosolic fraction. The final lane of the autoradiogram shows His-tagged huANT3 isolated from cell lysates using magnetic agarose beads coupled to Ni according to the manufacturers instructions (Qiagen; Hilden, Germany).

Thus, as in *E. coli*, huANT3 is expressed in the baculovirus/Sf9 system. Furthermore, recombinantly produced 6xHis- and epitope-tagged huANT3 fusion protein is appropriately localized to the mitochondria in Sf9 cells despite the presence of over 35 extraneous N-terminal amino acids, and can be isolated from cellular fractions by means that take advantage of the His-Tag moiety's affinity for metals such as, e.g., Nickel.

C. Baculovirus ANT Antisense Constructs

Because of their host specificity (i.e., beneficial insects are not infected), and their ability to persist in the environment, wildtype baculoviruses have been used as biological pesticides in some situations. Further advantages of the use of baculoviruses as biological control agents include their lack of environmental impact (as compared to, e.g, chemical pesticides) and the potential for their production, with a minimum of capital expense, in third world countries.

Although important differences exist in terms of structure and tissue expression patterns, ANT encoding sequences are very homologous with regards to isoforms within a species (compare the human ANT isoforms, *i.e.*, SEQ ID NOS:1,2 and 3) and also between species. (See, *e.g.*, the sequences of the *S. cerevisiae* AAC genes, which encode the three isoforms of the adenine nucleotide translocator present in yeast; Adrian et al., 1986, *Mol. Cell. Biol.* 6:626-634; Kolarov et al., 1990, *J. Biol. Chem.* 265:12711-12716; Lawson and Douglas, 1988, *J. Biol. Chem.* 263:14812-14818.) Accordingly, baculovirus huANT antisense expression constructs are expected to produce antisense RNAs having extensive stretches of complementarity to insect ANT mRNAs; such antisense RNAs will anneal to host ANT-encoding mRNAs *in vivo* and block their translation. Because ANT is an essential protein in most cells, as demonstrated by the toxicity of bongkreikic acid, loss of ANT function is expected to have toxic effects, thus enhancing the insect-killing capacity of wildtype baculovirus. Alternatively, the reverse complements of insect ANT cDNA sequences, or portions thereof that are strictly conserved in all isoforms present within a particular insect species, are used in such constructs.

As an example, a baculovirus huANT3 antisense expression construct is prepared in the following manner. DNA comprising nucleotide sequences encoding huANT3 is amplified by PCR from a whole human brain cDNA library (Clontech) using the following primers. In the following representations of PCR primers, underlined nucleotides indicate sequences complementary to the 5'-ends and 3'-ends of the ANT cDNAs and double-underlined nucleotides indicate recognition sequences for the restriction enzymes *Bam*HI (recognition sequence: 5'-GGATCC) or *Eco*RI (recognition sequence: 5'-GAATTC).

The PCR primers used are:

Reverse (sense):

5'-TTAAAGAGAATTCATGACGGAACAGGCCATCTCCTTCGCCAAA SEQ ID NO:26.

and Forward (antisense):

5'-TTATAGGATCCTTAGATCACCTTCTTGAGCTCGTCGTACAG SEQ ID NO:27.

PCR products are digested with the restriction endonucleases *Bam*HI (New England Biolabs) and *Eco*RI (New England Biolabs) according to the manufacturer's recommendations. Subsequent purification of the restricted PCR products is carried out by horizontal agarose gel electrophoresis and band extraction as described above.

The baculovirus transfer vector pBlueBacHis2 is prepared by digestion with the restriction endonucleases *Bam*HI and *Eco*RI, and the restricted DNA subject to horizontal agarose gel electrophoresis and band extraction, as previously described. The restricted PCR products are ligated with the restricted plasmid DNA, and competent *E. coli* cells transformed with the ligation reactions, as in the preceding Examples. Single colonies are selected for growth in 3-5 ml of LB broth containing 50 ug/ml, and plasmid DNA is isolated from the bacterial cultures.

The structure of baculovirus antisense transfer vectors are confirmed by sequence analyses, and inserted into the baculoviral genome, as described in the preceding section. Baculovirus ANT antisense expression constructs are screened for their insecticidal efficacy using, for example, the Diet Plug Assay or the Droplet Feeding Assay (Cory and Bishop, Chapter 16 in: *Baculovirus Expression Protocols*, Methods in Molecular Biology, Vol. 39, C.R. Richardson, Ed., Humana Press, Totawa, NJ, 1995, pages 277-294, and references cited therein). Means for the formulation and application of baculovirus for insecticidal purposes are known in the art (*Id.*) and are adapted for baculovirus ANT antisense expression constructs.

EXAMPLE 4

EXPRESSION OF ANT3 IN YEAST

A. Expression Constructs and Host Cells

Human ANT3 cDNA was amplified by PCR as in Example 1 but using the following primers. In the following representations of PCR primers, underlined nucleotides indicate sequences complementary to the 5'-ends and 3'-ends of the ANT

cDNAs and double-underlined nucleotides indicate recognition sequences for the restriction enzymes *Xho*I (recognition sequence: 5'-CTCGAG) or *Asp*718 (recognition sequence: 5'-GGTACC).

The primers used for PCR amplification were:

- 5 Forward (sense; SEQ ID NO:28):

5'-TTAATGGGTACCATGACGGAACAGGCCATCTCCTTCGCCAAA, and

Reverse (antisense; SEQ ID NO:29):

5'-TTATACTCGAGTTAGATCACCTTCTTGAGCTCGTCGTACAGG.

- PCR products, and expression vector DNAs, were digested with the
10 restriction endonucleases *Xho*I and *Asp*718 (both enzymes from Roche Molecular Biochemicals) according to the manufacturer's recommendations using manufacturer-supplied reaction buffers. The expression vector pYES2 (Invitrogen) was used. This vector contains a multiple cloning site located immediately downstream from an inducible *GAL*I promoter, as well as the 2u origin of replication and the *S. cerevisiae*
15 *URA3* gene for high-copy maintenance and selection in *ura3* yeast cells, respectively.

- The restricted DNAs were purified by horizontal agarose gel electrophoresis and band extraction using the UltraClean GelSpin kit (Mo Bio Laboratories), ligated to each other, and used to transform *E. coli* cells, as in the preceding Examples. Plasmid DNA was isolated from several transformants, and the
20 nucleotide sequence of the insert DNA was determined and confirmed to be that of huANT3. One confirmed plasmid was chosen to be used for further study and was designated pMK5A (huANT3).

- A second yeast huANT3 expression vector, pMK5B, was constructed as follows. Plasmids pMK5A and pYESTrp2 (Invitrogen) were digested with restriction
25 enzymes *Bgl*II and *Pvu*II (both from New England Biolabs) and gel purified, ligated and used to transform *E. coli* as above. The expression vector pYES2Trp is similar to pYES2 but comprises a *TRP*I selectable marker. Plasmid DNA was isolated from several transformants and restriction mapped to confirm the structure of the expected expression construct. One confirmed plasmid was chosen to be used for further study
30 and was designated pMK5B (huANT3).

A third yeast huANT3 expression vector, pMK5C, was constructed using the expression vector pYPGE2, which comprises a *TRP1* selectable marker and the strong *PGK* promoter upstream from a multiple cloning site (Brunelli and Pall, 1993 *Yeast* 9:1299-1308). Plasmid pYPGE2 DNA was digested with *Xho*I and *Asp*718, gel-purified and ligated with the *Xho*I- and *Asp*718-restricted huANT3 PCR product of Example 1. The ligation mixture was used to transform *E. coli*, and plasmid DNA was isolated from several transformants and restriction mapped to confirm the structure of the expected expression construct. One confirmed plasmid was chosen to be used for further study and was designated pMK5C (huANT3).

In order to generate yeast expression systems, the *S. cerevisiae* strain INVSc1 (*MAT* α , *his3* Δ 1, *leu2*, *trp1*-289, *ura3*-52) was transformed with purified pMK5A, pMK5B and pMK5C DNAs using the *S.c.* EASYCOMP™ Transformation Kit (Invitrogen). A second *S. cerevisiae* strain, J Δ 1 Δ 3 (*MAT* α , *ade2*-1, *leu2*-3, *leu2*-112, *his3*-11, *his3*-15, *trp1*-1, *ura3*-1, *can1*-100, *AAC1*::*LEU2*, *AAC2*::*HIS3*, *AAC3*::*URA3*) was also transformed with the expression constructs. The AAC genes encode the three isoforms of the mitochondrial ADP/ATP translocator in *S. cerevisiae* and are interrupted in strain J Δ 1 Δ 3 (Giraud et al., *J. Mol. Biol.* 281:409-418 (1998)). It is thus expected that transformants of J Δ 1 Δ 3, which are incapable of expressing endogenous ANT (AAC) proteins, will only express the human ANT protein encoded by the expression construct with which they have been transformed.

B. Northern blot analyses of yeast expression systems

In order to examine levels of huANT3 mRNA production in strain J Δ 1 Δ 3, Northern analyses of cells transformed with pMK5B and pMK5C were performed according to methods known in the art. In brief, transformed cells and control (untransformed) cells grown to mid-log phase, harvested and lysed. RNA was extracted from the lysates, electrophoresed and transferred to a nitrocellulose filter (*see* Treco, Preparation of Yeast RNA, Unit 13.12 of Chapter 13 in *Short Protocols in Molecular Biology*, 2nd Ed., Asubel et al., eds., John Wiley & Sons, New York, New York (1992), 13:44-46 and Seldon, *Analysis of RNA* by Northern Hybridization, Unit

4.9 of Chapter 4, *Id.*, 4:23-25). The *Xho*I- and *Asp*718-restricted huANT3 PCR product of Example 1 was radiolabelled and used as a probe, and an RNA preparation from human spleen tissue was used as a positive control.

The results (Figure 10) demonstrate the appropriately-sized ANT3-specific RNA is produced in human spleen and in yeast cells transformed with either expression vector, but not in untransformed yeast cells. The pYPGE2-derived expression construct pMK5C, which directs ANT3 expression from the *PGK* promoter, clearly results in more ANT3 RNA than the pYES2Trp-derived construct pMK5B, in which ANT3 expression is driven by the *GALI* promoter. In either case, however, significant levels of huANT3-specific RNA was produced in a yeast background that lacks any endogenous adenosine nucleotide translocator proteins.

C. Western Analyses of Yeast Expression Systems

1. Production of antibody to huANT3

As the huANT3 produced from the yeast expression constructs lacks an epitope tag, it was necessary to produce an antibody to huANT3 in order to evaluate recombinant production of the protein. A nonspecific (anti-peptide) antibody specific to huANT3 was prepared as follows.

A synthetic polypeptide corresponding to a portion of huANT3 located near the carboxy terminus and predicted to have high antigenicity according to the Jameson-Wolf Index (Wolf et al., *Comput. Appl. Biosci.* 4:187-191 (1988)) was synthesized using known means by Alpha Diagnostic International (San Antonio, TX) and determined to be at least about 70% pure, preferably at least about 90% pure, by HPLC and MS analyses. The sequence of the synthetic polypeptide (SEQ ID NO:30) is:

Cys-Trp-Arg-Lys-Ile-Phe-Arg-Asp-Glu-Gly-Gly-Lys-Ala-Phe-Phe

The synthetic polypeptide was conjugated to a carrier molecule, keyhole limpet hemocyanin (KLH), using MSB (*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; Pierce Chemical Co., Rockford, Illinois), and the conjugated material was used to immunize several rabbits, according to known means (Collawn and Paterson, Units

11.14 and 11.15 in Chapter 11 in: *Short Protocols in Molecular Biology*, 2nd Ed., Asubel et al., eds., John Wiley & Sons, New York, New York (1992) 11:37-41. The rabbits were or are bled at 0 (preimmune, 2 mL), 7, 9, 11, 13 (15 mL for each bleed) or 15 weeks (50 mL) post-inoculation. Sodium azide (0.1%) was or is added to the bleeds as preservative.

2. Western analyses

Western analyses of yeast expression systems are performed essentially as described in the preceding Examples, except that different methods are used to prepare protein preparations from yeast cells as opposed to bacterial or insect cells.

Such methods of isolating proteins from yeast are known in the art (*see, for example*, Dunn and Wobbe, Preparation of Protein Extracts from Yeast, Unit 13.13 of Chapter 13 in *Short Protocols in Molecular Biology*, 2nd Ed., Asubel et al., eds. John Wiley & Sons, New York, New York (1992), 13:46-50). The intracellular distribution of huANT3 in, *e.g.*, membrane or mitochondrial fractions, is determined as in the preceding Examples.

EXAMPLE 5

EXPRESSION OF ANT3 IN MAMMALIAN CELLS

The preceding Examples describe a variety of means by which ANT and ANT fusion proteins can be recombinantly produced in various systems. Although such ANT proteins can be used in a variety of assays (*see infra*), it may be desirable to isolate large amounts of the native ANT protein from mammalian cells. In particular, as described in this Example, it may be desirable to produce recombinant viral particles in which ANT proteins are displayed in the viral envelope. Such ANT-displaying viral particles are expected to be very stable and useful in a variety of assays including, for example, those in which compounds binding to ANT proteins are screened and identified.

Another useful outcome of mammalian expression systems is the generation and isolation of human mitochondria in which a particular ANT isoform is

over-represented in order to determine the specific biological role(s) of such isoforms. For example, ANT3 is apparently ubiquitously expressed in human tissues, whereas ANT1 is primarily expressed in heart and skeletal muscle (Stepien *et al.*, 1992, *J. Biol. Chem.* 267:14592-14597). Directed overexpression of huANT1 in cultured heart or muscle cells is expected to result in mitochondria that contain mostly the ANT1 isoform. Such “ANT isoform-enriched” mitochondria can be isolated and tested for various mitochondrial functions.

Constructs for expressing ANT proteins in mammalian cells are prepared in a stepwise process. First, expression cassettes that comprise a promoter (and associated regulatory sequences) operably linked to nucleotide sequences encoding an ANT protein are constructed in bacterial plasmid-based systems; these expression cassette-comprising constructs are evaluated and optimized for their ANT-producing ability in mammalian cells that are transiently transfected therewith. Second, the ANT expression cassettes are transferred to viral systems that produce recombinant proteins during lytic growth of the virus (e.g., SV40, BPV, EBV, adenovirus; see below) or from a virus that can stably integrate into and transduce a mammalian cellular genome (e.g., a retroviral expression construct).

A. Transient expression

With regards to the first step, commercially available “shuttle” (i.e., capable of replicaton in both *E. coli* and mammalian cells) vectors that comprise promoters that function in mammalian cells and can be operably linked to an ANT-encoding sequence include, but are not limited to, SV40 late promoter expression vectors (e.g., pSVL, Pharmacia), glucocorticoid-inducible promoter expression vectors (e.g., pMSG, Pharmacia), Rous sarcoma enhancer-promoter expression vectors (e.g., pRc/RSV, Invitrogen) and CMV early promoter expression vectors, including derivatives thereof having selectable markers to agents such as Neomycin, Hygromycin or ZEOCINTM (e.g., pRc/CMV2, pCDM8, pcDNA1.1, pcDNA1.1/Amp, pcDNA3.1, pcDNA3.1/Zeo and pcDNA3.1/Hygro, Invitrogen) In general, preferred shuttle vectors for ANT genes are those having selectable markers (for ease of isolation and

maintenance of transformed cells) and inducible, and thus regulatable, promoters (as overexpression of ANT genes may have toxic effects).

Methods for transfecting mamallian cells are known in the art (see, Kingston *et al.*, "Transfection of DNA into Eukaryotic Cells," Section I of Chapter 9 in: *Short Protocols in Molecular Biology*, 2nd Ed., Asubel *et al.*, eds., John Wiley & Sons, New York, New York, 1992, pages 9-3 to 9-16). A control plasmid, such as pCH110 (Pharmacia), may be cotransfected with the ANT expression construct being examined so that levels of ANT can be normalized to a gene product expressed from the control plasmid.

Western analyses of mammalian expression systems are performed essentially as described in the preceding Examples, except that different methods are used to prepare protein preparations from mamallian cells as opposed to bacterial, insect or yeast cells. Such methods of isolating proteins from yeast are known in the art (see, for example, Kingston and Sheen, Unit 9.6A and Brasier, Unit 9.6B of Chapter 9 in: *Short Protocols in Molecular Biology*, 2nd Ed., Asubel *et al.*, eds., John Wiley & Sons, New York, New York, 1992, pages 9-17 to 9-23). Preferred expression cassettes, consisting essentially of a promoter and associated regulatory sequences operably linked to an ANT gene of interest, are identified by the ability of cells transiently transformed with a vector comprising a given ANT expression cassette to express high levels of ANT protein when induced to do so; these expression cassettes are incorporated into viral expression vectors.

B. Viral expression

Nucleic acids, preferably DNA, comprising preferred expression cassettes are isolated from the transient expression constructs in which they were prepared, characterized and optimized (see preceding section). A preferred method of isolating such expression cassettes is by amplification by PCR, although other methods (e.g., digestion with appropriate restriction enzymes) can be used. Preferred expression cassettes are introduced into viral expression vectors, preferably retroviral expression vectors, in the following manner.

A DNA molecule comprising a preferred expression cassette is introduced into a retroviral transfer vector by ligation (see preceding Examples). Two types of retroviral transfer vectors are known in the art: replication-incompetent and replication-competent. Replication-incompetent vectors lack viral genes necessary to produce infectious particles but retain *cis*-acting viral sequences necessary for viral transmission. Such *cis*-acting sequences include the Ψ packaging sequence, signals for reverse transcription and integration, and viral promoter, enhancer, polyadenylation and other regulatory sequences. Replication-competent vectors retain all these elements as well as genes encoding virion structural proteins (typically, those encoded by genes designated *gag*, *pol* and *env*) and can thus form infectious particles in a variety of cell lines. In contrast, these functions are supplied in *trans* to replication-incompetent vectors in a packaging cell line, i.e., a cell line that produces mRNAs encoding *gag*, *pol* and *env* genes but lacking the Ψ packaging sequence. See, generally, Cepko, Unit 9.10 of Chapter 9 in: *Short Protocols in Molecular Biology*, 2nd Ed., Asubel *et al.*, eds., John Wiley & Sons, New York, New York, 1992, pages 9-30 to 9-35.

A retroviral construct comprising an ANT expression cassette produces RNA molecules comprising the cassette sequences and the Ψ packaging sequence. These RNA molecules correspond to viral genomes that are encapsidated by viral structural proteins in an appropriate cell line (by “appropriate” it is meant that, for example, a packaging cell line must be used for constructs based on replication-incompetent retroviral vectors). Infectious viral particles are then produced, and released into the culture supernatant, by budding from the cellular membrane. The infectious particles, which comprise a viral RNA genome that includes the ANT expression cassette, are prepared and concentrated according to known methods. It may be desirable to monitor undesirable helper virus, *i.e.*, viral particles which do not comprise an ANT expression cassette. See, generally, Cepko, Units 9.11, 9.12 and 9.13 of Chapter 9 in: *Short Protocols in Molecular Biology*, 2nd Ed., Asubel *et al.*, eds., John Wiley & Sons, New York, New York, 1992, pages 9-36 to 9-45.

Viral particles comprising an ANT expression cassette are used to infect *in vitro* (e.g., cultured cells) or *in vivo* (e.g., cells of a rodent, or of an avian species,

which are part of a whole animal). Tissue explants or cultured embryos may also be infected according to methods known in the art. See, generally, Cepko, Unit 9.14 of Chapter 9 in: *Short Protocols in Molecular Biology*, 2nd Ed., Asubel *et al.*, eds., John Wiley & Sons, New York, New York, 1992, pages 9-45 to 9-48. Regardless of the type of cell used, production of ANT protein is directed by the recombinant viral genome.

In a preferred embodiment, recombinantly produced ANT proteins are inserted into the cell membrane of cultured cells. Because the retroviral expression construct produces viral particles by budding of the cell membrane, the resultant viral particles delivered to the culture supernatant have ANT protein incorporated into their capsules, preferably on the surface of the particles. Such ANT-displaying viral particles are expected to provide a stable format for ANT proteins and to thus be useful in assays using ANT proteins, either directly or as a source material from which ANT can be further purified. If it is desired to minimize the amount of ANT protein inserted into mitochondrial membranes, ρ^0 cells, which have been treated in such a manner as to be nearly or completely devoid of mitochondria, are used as host cells.

C. ANT Antisense Constructs

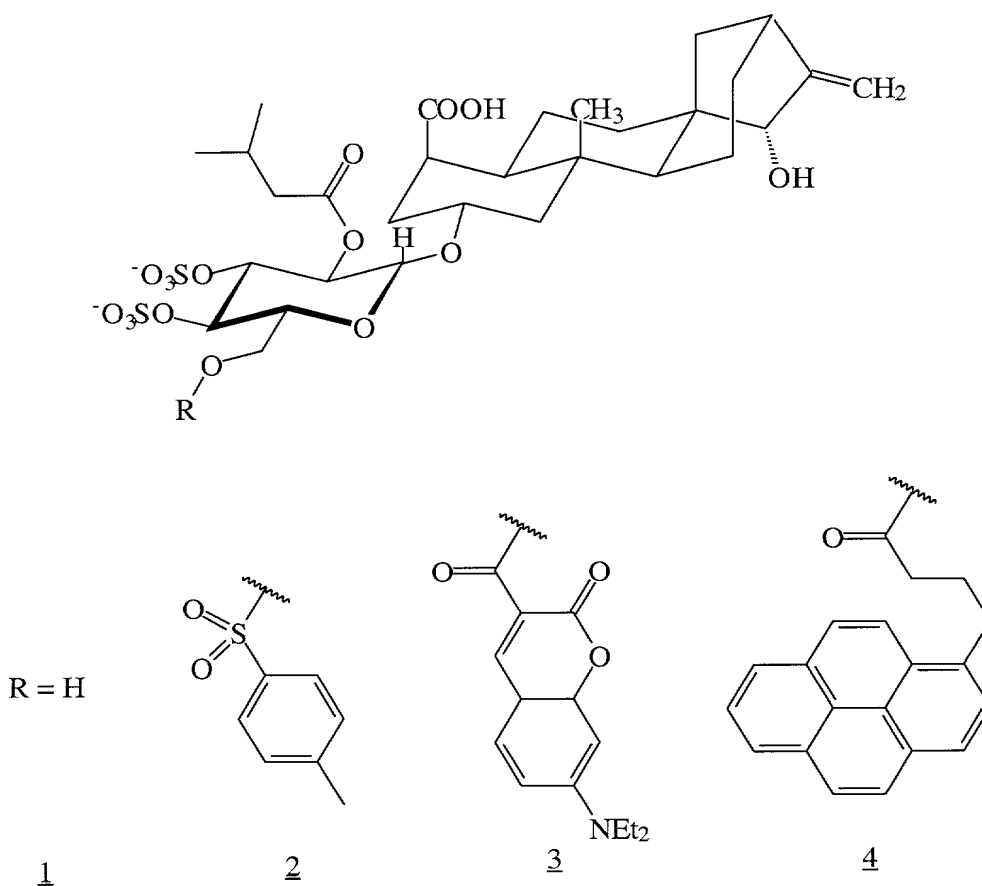
Antisense versions of the preceding transient and viral ANT expression constructs are prepared by exchanging the antisense (non-encoding) strand for a sense (ANT protein encoding) strand in a construct. Such ANT antisense constructs are useful as research reagents, i.e., to reduce levels of expression of one or more isoforms in a cell transformed or infected with such a construct in order to determine the effects of such treatment on cellular physiology. ANT antisense constructs are also useful as gene therapy agents that interfere with the translation of one or more isoforms of ANT.

EXAMPLE 6

SYNTHESIS AND PROPERTIES OF REPRESENTATIVE ATR DERIVATIVES

A number of atractyloside (ATR) derivatives were prepared for use as ligands for adenine nucleotide translocators (ANTs) in the context of high-throughput screening assays. These compounds bind with high affinity (*i.e.*, in the nM range) to

ANT and are thus useful for screening libraries of chemical compounds for molecules having high specificity for ANT (regardless of isoform). The structure of ATR is set forth below as compound (1). Compounds (3) and (4) represent novel fluorescent derivatives of ATR, while compound (2) is an ATR derivative which permits introduction of the ^{125}I under mild conditions.



Purification

Compounds 2, 3 and 4 were purified by silica gel chromatography using $\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{AcOH}$ (75:25:1) as the eluting solution. Detection was achieved by staining with a 0.5% solution of vanillin in $\text{H}_3\text{PO}_4/\text{H}_2\text{O}$ (1/1). Further purification was accomplished by reversed-phase HPLC using a Microsorb C8 column (250 × 10 mm). The column was eluted at a flowrate of 2.0 mL/min with a linear gradient of methanol/acetic acid/1 M ammonium acetate 98:1:1 ("Solvent B") in $\text{H}_2\text{O}/\text{acetic acid}/1$

M ammonium acetate 98:1:1 ("Solvent A"). The effluent was monitored for absorbance at 254 nm. Compound-containing fractions were pooled, evaporated, and repeatedly co-evaporated with added methanol (3x 5 mL).

Synthesis of Compound 2

- 5 Atractyloside 1 (0.10 mmol) was dried by repeated evaporation of added pyridine (3x 5 mL) and the resulting gummy residue dissolved in pyridine (5 mL). To the resulting solution, 0.20 mmol of toluenesulfonyl chloride was added. The reaction mixture was stirred at ambient temperature for 1.5 h. Then, another portion of toluenesulfonyl chloride (0.20 mmol) was added and the reaction left stirring an
10 additional 1.5 h. 1 mL of methanol was added to the reaction mixture which was then stirred for 0.5 h, after which solvents were removed by evaporation. Residual pyridine was removed by evaporation of additional methanol (5x 10 mL). Silica gel chromatography followed by reversed-phase HPLC using a linear gradient of 50-80% of solvent B in solvent A for 30 min. resulted in the compound 2 eluting at 68% solvent B.
15 Yield: 4.3 mg, 4.9%. ESI-MS (M-H) found:879, calc.:879.

Synthesis of Compounds 3 and 4

- 7-Diethylamino-2-oxo-2*H*-chromene-3-carboxylic acid or 0.20 mmol of 1-pyrenebutyric acid and 0.60 mmol of 1,1'-carbonyldiimidazole in 1 mL of dimethylformamide were allowed to react for 15 min. To the activated carboxylic acid
20 was added a solution of atractyloside 1 in H₂O (4 mL) and the resulting reaction mixture was stirred at ambient temperature for 16 h. Evaporation left a gummy residue which was purified by silica gel chromatography followed by reversed-phase HPLC. Using a linear gradient of 10-80% of solvent B in solvent A for 50 min (for compound 3) or 50-100% of solvent B in solvent A for 50 min (for compound 4) resulted in compound 3
25 eluting at 75% B and compound 4 eluting at 82% B. Yields: compound 3, 3.1 mg, 8.0%; compound 4, 1.3 mg, 3.6%. ESI-MS (M-H) compound 3 found:968, calc.: 968; compound 4 found:995, calc.:995.

Properties of Representative ATR Derivatives

As summarized in Table 1 below, compounds 3 and 4 were found to be more advantageous in terms of fluorescence characteristics and sensitivity compared to the existing ATR derivatives Naphthoyl-ATR and MANT-ATR as reported by Boulay et al., *Analytical Biochemistry* 128:323-330,1983; Roux et al., *Analytical Biochemistry* 234:31-37,1996; Lauquin et al., *FEBS Letters* 67:306-311,1976; and Gottikh et al., *Tetrahedron* 26:4419-4433, 1970.

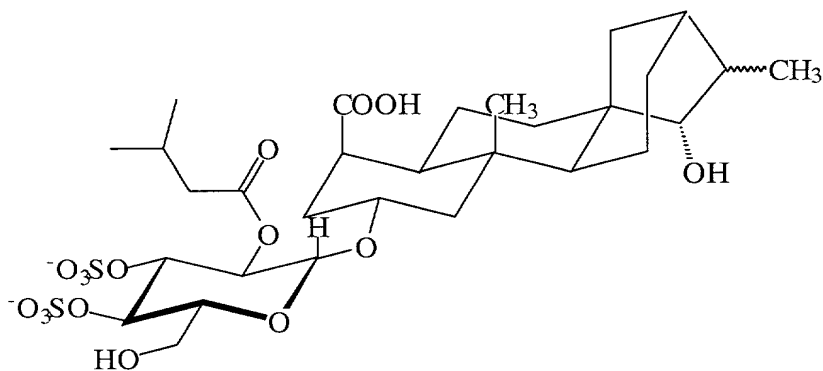
TABLE 1

ATR Derivative	Excitation (nm)	Emission (nm)	Extinction Coefficient (M^{-1}) (Predicted)
Naphthoyl-ATR	300	405	6,200
MANT-ATR	350	460	5,800
Compound <u>4</u>	341	391	17,420
Compound <u>3</u>	417	470	46,400

EXAMPLE 7

SYNTHESIS OF REPRESENTATIVE ATR DERIVATIVE

The further representative ATR derivative, compound 5, was prepared by the procedure set forth below.



5

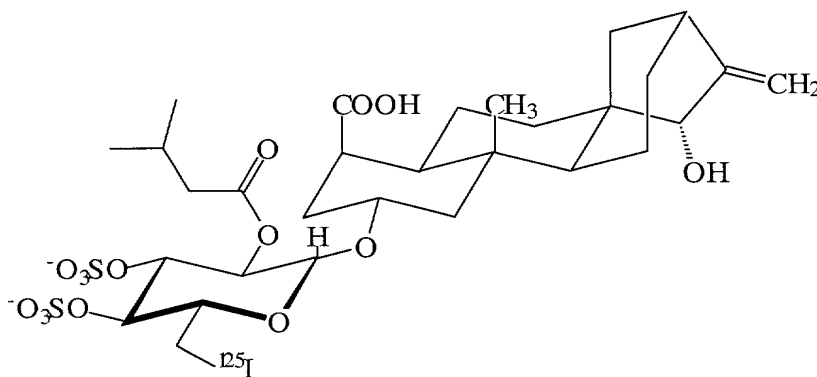
Synthesis of Compound 5

Dipotassium atractylate (0.10 mmol) was dissolved in 50% aq. ethanol (5 mL) and palladium on charcoal (10%, 17 mg) was added to the reaction mixture. After flushing the system with argon, the reaction mixture was stirred under an atmosphere of hydrogen gas for 3 h. Removal of catalyst by filtration through Celite, washing with 50% aq. ethanol (10 mL), and evaporation of solvents afforded a white solid. Yield after thorough drying under high vacuum; 78.3 mg (97.3%). ESI-MS (M-2H+K) found:765, calc.:765. ¹H-NMR analysis confirmed the absence of alkenic protons. Compound 5 was found to have equivalent affinity for ANT as atractyloside. Thus, hydrogenation in the presence of tritium will provide a tritiated derivative that can be utilized as a radioactive ANT ligand in various binding assays.

EXAMPLE 8

SYNTHESIS OF REPRESENTATIVE IODINATED ATR DERIVATIVE

Compound 2 of Example 6 may be used as intermediate for conjugation of variety of chemical moieties to yield further ATR derivatives. In this example, compound 2 is employed to introduce ¹²⁵I under mild conditions to yield the following compound 6.



Synthesis of Compound 6

Five μ l of 0.2 M sodium phosphate (pH 5) was combined with 21 μ l of Na^{125}I (9.25 mCi) in its shipping container (specific activity, 2100 Ci/mmol; Amersham, Piscataway, New Jersey). Ten μ l (200 μ g, 212 nmol) of compound 2 of Example 1 was added to the mixture. The pH was checked with litmus paper to confirm that it did not rise above pH 5. The mixture was allowed to stand at ambient temperature overnight (17.5 hours) to yield radiolabelled compound 6. (Non-radioactive iodinated ATR derivative, for use as a "cold" competitor in binding studies, may be prepared in the same manner using unlabeled iodine). The iodinated derivative was purified over a C18 analytical column (4 x 6 x 250 mm) (Phenomenex, Torrance, California) using a 25%-55% acetonitrile gradient in running buffer (1% triethylammonium acetate, pH 4.5). A flow rate of 1 ml/min was used to run the gradient over 30 min. The desired product eluted at 25 min.

EXAMPLE 9

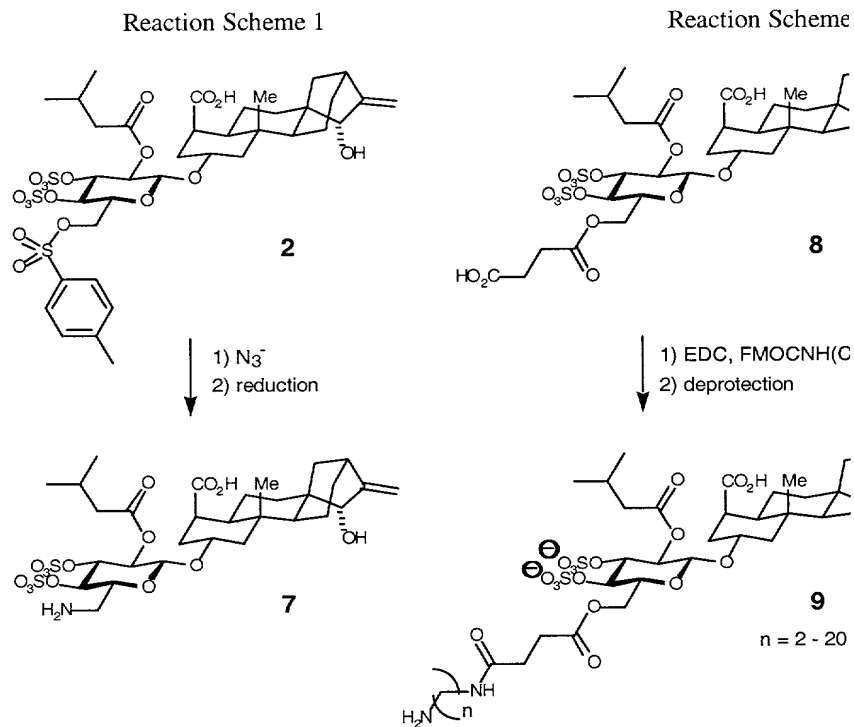
SYNTHESIS OF REPRESENTATIVE ATR DERIVATIVES

Activation of carboxylic acids with carbonyl diimidazole and their reaction with ATR has been the method of choice for synthesis of various 6'-O-acyl derivatives. The relatively low reactivity of the 6'-hydroxyl of ATR and the presence of an allylic secondary hydroxyl in the aglycon as well as the sulfated glucose moiety, are all factors that have a negative impact on the efficiencies of these acylation reactions. Hence, yields are generally poor and the approach requires a large excess of acylating reagents.

Two strategies for introduction of an amine functionality in the ATR system are described below that permit synthesis of a broader range of ATR derivatives. In the first strategy, as depicted by Scheme 1, displacement of the primary tosylate from compound 2 (Example 1) with azide followed by reduction yields the corresponding 6'-amine (compound 7). Alternatively, the amine group can be introduced as part of a

spacer, which permits introduction of more sterically demanding functional moieties. Thus, reacting the 6'-O-succinoyl derivative (compound 8; *see* Brandolin et al., 1974 *FEBS Lett.* 46:149.) with a monoprotected diamine followed by deprotection affords compound 9 as illustrated by Reaction Scheme 2.

5



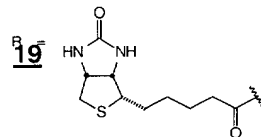
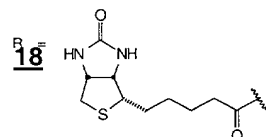
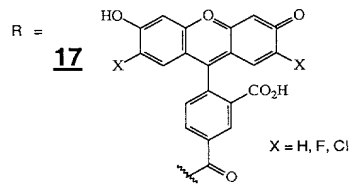
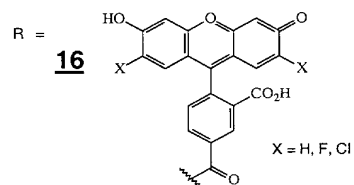
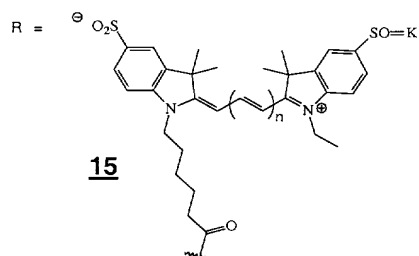
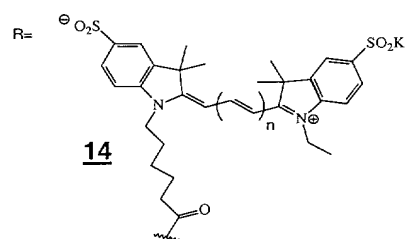
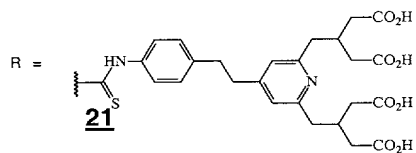
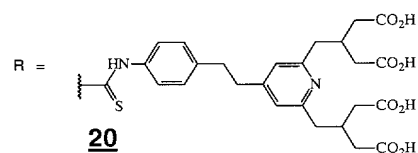
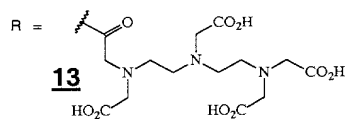
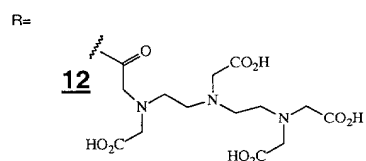
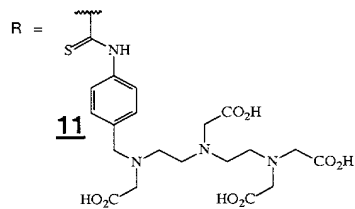
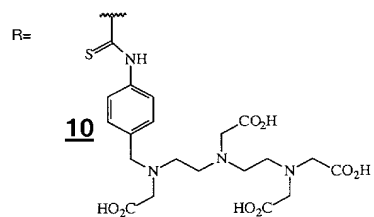
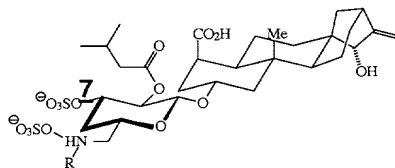
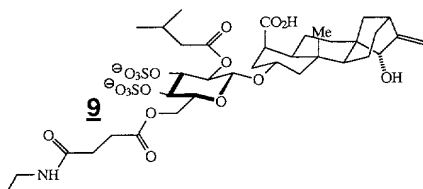
The amine-containing ATR derivatives 7 and 9 may then be reacted with a variety of fluorophors and haptens bearing reactive isothiocyanate, N-hydroxysuccinimide ester and anhydride functionalities to yield stable ATR-derivatives having thiourea and amide linkages. Representative ATR derivatives that were prepared include ATR-lanthanide chelating agents (compounds 10, 11, 12, 13, 20 and 21) that have utility for time-resolved fluorescence detection of these compounds complexed to Eu^{2+} . In addition, ATR was conjugated to cyanine (compounds 14 and 15) and fluorescein analogues (compounds 16 and 17) that are detectable by fluorescence with extremely high sensitivities. Coupling of biotin-NHS ester with the ATR derivatives of compounds 7 and 9 provided ATR-biotin conjugates (compounds 18 and 19) that can be detected with commercially available enzyme-avidin conjugates using colorimetric, fluorescent or chemiluminescent techniques.

More specifically, a solution of compound 2 in DMF was treated with azide ion for 8 hours at 80°C to give the 6'-azido-ATR, that was purified by silica gel chromatography using a $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ solvent system supplemented with 1% acetic acid. Staudinger-reduction using 1.5 equivalents of triphenylphosphine in a $\text{THF}/\text{H}_2\text{O}$ mixture for 4 hours at RT afforded the amine of compound 7, that was isolated after silica gel chromatographic purification.

To accommodate more sterically demanding functional moieties, 6'-O-succinoyl-ATR may be condensed with commercially available monoprotected diamines (Calbiochem-Novabiochem Corp, San Diego, CA) to produce ATR-monoprotected amine derivatives. Thus, EDC-mediated coupling of 6'-O-succinoyl-ATR in DMF with 1.1 equivalents of mono-protected Fmoc diamines yield the amide that was deprotected using piperidine or 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in acetonitrile to furnish the ATR derivative of compound 9. The amines were purified by silica gel chromatography as described above.

The ATR-amine derivatives of compounds 7 and 9 were coupled to a variety of fluorophors, chelates and haptens that contained amine-reactive functionalities, such as isothiocyanates, anhydrides and NHS esters in aqueous DMF to

generate the ATR derivatives of compounds 10 through 19: These compounds were purified by a combination of silica gel chromatography and preparative reverse phase chromatography on a C-8 column using CH₃OH/H₂O gradient containing 0.1-1% acetic acid.



EXAMPLE 10

BINDING ASSAYS USING RECOMBINANT HUANT3

A. GST-huANT3 Recombinantly Produced in *E. coli*:

5 Following arabinose induction, transformed or sham transformed (vector only) *E. coli* were collected by centrifugation at 2000 g for 10 min. The bacterial pellets were resuspended in MSB, to which lysozyme (100 µg/ml) was added. After 20 min at room temperature, the lysates were subjected to one freeze-thaw cycle followed by sonication as described above. The resultant membrane preparation was used for
10 binding assays.

 To estimate maximal binding and the extent of overexpression of the huANT3, 25 µg of membrane protein was incubated with varying amounts of [³²P]ATP (1-500 µM) in binding buffer (120 mM KCl, 10 mM Tris, 1 mM EDTA, pH 7.4) for 2 hr at room temperature. The membranes with bound ATP were sedimented by
15 centrifugation at 5000 g for 5 min, and washed once with binding buffer. Membrane pellets were then mixed with 5 ml scintillation cocktail and counted. The results are presented in Table 2.

Table 2: Saturation Binding of [32 P]ATP to *E. coli* Membranes

5	[ATP], μ M	cpm bound by:	
		Sham Transformed Cells	huANT3-Producing Cells
10	0.1	109	191
	0.5	95	49
	1.0	147	325
	5.0	123	N.D.
	10	214	263
	50	549	2,727
	100	718	5,772
	500	2,140	9,715

15 N.D., not determined

The data presented in Table 2 indicate that the affinity of the ATP binding was $\sim 6 \mu$ M. ATP binding was completely abolished by the addition of atracyloside (10μ M) to the assay. These results support the contention that the measured ATP binding was predominantly to recombinantly produced ANT3 protein.

Agarose-glutathione beads were incubated with solubilized (using Dnase, Rnase and 0.1% Triton X-100; see Example 1, section D) bacterial lysate (see Example 2), and substituted for the *E. coli* membranes in binding assays. Best results (*i.e.*, more specific binding) were seen when the beads were preincubated with bovine serum albumin (BSA, 0.1%). Specific ATP binding (1,070 cpm) was also observed in this experiment (compare to nonspecific binding of 279 cpm in the presence of 10 mM non-radiolabeled ATP).

B. huANT3 from a Sf9/Baculovirus Expression System

Sf9 cells expressing huANT3 were grown in spinner flasks. The cells were harvested by centrifugation at 2,000 g for 5 min. The cell pellet was resuspended in MSB and subjected to 3 freeze-thaw cycles. Cell membranes and debris were removed by centrifugation at 600 g for 5 min; mitochondria were collected by centrifuging the supernatant at 20,000 g for 15 min. The mitochondrial pellets were

suspended in MSB, and used for binding assays as described above. Homologous competition of [32 P]ATP binding was performed using 25 μ g mitochondrial protein per assay.

As illustrated in Figure 7, ATP bound to the mitochondria with $K_d = 13$ μ M, a value consistent with ATP binding to ANT. Furthermore, the ATP binding was displaced by low concentrations of atractyloside (Figure 8). Homologous competition binding assays using [125 I]atractyloside revealed specific binding with $K_d = 12$ nM (Figure 9). These findings are consistent with the presence of functional huANT3 in the mitochondrial preparations.

His-tagged huANT3 protein was purified from baculovirus-infected Sf9 solubilized cell lysates using Ni-agarose magnetic beads; Sf9 cells that had not been infected were used as negative controls. The beads were incubated with [32 P]ATP (1 or 100 μ M) for 2 hr. The beads were washed and then counted to determine the amount of bound ATP. As shown in Table 3, the [32 P]ATP binding was significantly higher in material recovered from the infected cells than in the controls. Binding saturation had essentially been achieved with 1 μ M ATP.

Table 3: Binding of [32 P]ATP to Purified His-Tagged huANT3

<u>[ATP], μM</u>	<u>cpm bound by:</u>	
	<u>Control (Uninfected) Cells</u>	<u>huANT3-Producing Cells</u>
1.0	43	149
100	30	160

EXAMPLE 11

COMPETITIVE BINDING ASSAYS

Atractyloside analogs (Example 6; Table 1; see also Examples 7-9) were used in pseudo-homologous competition binding assays using Sf9/huANT3 mitochondria. Mitochondria (25 μ g/tube) were incubated with 0.5 nM [125 I]attractyloside and varying concentrations of non-radiolabeled attractyloside or fluorescent attractyloside derivatives as described above (Figures 7-9).

The results (Table 4) show that three of the attractyloside derivatives (MANT-, Pyrene- and Coumarin-attractyloside) had relative binding affinities similar to that of authentic attractyloside ($IC_{50} < 500$ nM relative to attractyloside). Each of these derivatives is fluorescent, and may therefore be useful as detectable ligands for binding assays.

Table 4: Competitive Binding Assays Using [125 I]Atractyloside

		cpm [125 I]Atractyloside bound in the presence of:			
	[ATR Derivative], nM	ATR	COU-ATR	PYR-ATR	MANT-ATR
	0.0	227	437	437	437
	1.0	224	391	350	---
	5.0	146	---	---	---
	10	42	371	349	229
	50	26	---	---	---
	100	---	277	362	195
	1,000	36	174	238	---
	5,000	45	---	---	---
	10,000	0	100	0	---

Abbreviations and symbols:

ATR, attractyloside.

COU-ATR, Coumarin-atractyloside, (Table 1, compound 3).

PYR-ATR, Pyrene-atractyloside, (Table 1, compound 4).

MANT-ATR, MANT-atractyloside, (Table 1, Roux et al. 1996 *Anal. Bioch.* 234:31)

5 ---, not determined.

EXAMPLE 12

HIGH THROUGHPUT SCREENING ASSAY FOR COMPOUNDS TARGETED TO ANT PROTEINS AND POLYPEPTIDES

10 The recombinantly produced ANT proteins, ANT fusion proteins and detectably labeled ANT ligands described herein are incorporated into automated assay systems. Such automated systems are useful for high throughput screening (HTS) of candidate ANT-binding compounds or chemical libraries comprising such compounds.

15 Such compounds may be further characterized and developed as drug candidates and drugs useful for preventing, treating or curing diseases or disorders resulting from the overexpression or dysfunction of one or more ANT proteins or from the overexpression or dysfunction of a factor that positively regulates or stimulates ANT proteins.

A preferred element of many automated assay systems is the
20 incorporation of a target molecule (in the present instance, an ANT protein) into a 96-well plate. This format is readily adaptable for use in a variety of automated label detection systems. For HTS assays, robotic label detection systems are preferred.

As one example of an HTS comprising the elements describes herein, the
GST-huANT3 fusion protein of Example 2 is contacted with REACTI-BIND™
25 glutathione-coated 96-well plates (Pierce). Glutathione coated strip-well plates are preferably used for assays comprising radiolabeled ANT ligands (e.g., iodinated atractyloside derivatives; see Example 7), whereas black opaque glutathione coated 96-well plates are preferred for assays comprising fluorescent ANT ligands (such as are described in, e.g., Examples 6-9); both types of glutathione coated plates are
30 commercially available (Pierce).

In a typical assay, 1 to 50 ug of GST-huANT3 protein (i.e., total solubilized protein prepared as in Example 2) is added per glutathione-coated well to each well of a 96-well plate. Iodinated atractyloside derivate (^{125}I -ATR) is added to the wells (0.5 nmol/well). In a control experiment, unlabeled atractyloside (ATR; Sigma) is used as a 'mock' drug at a concentration of from about 1 to about 10,000 nM. That is, unlabeled ATR is used to displace a labeled atractyloside derivative (e.g., ^{125}I -ATR). Unlabeled ATR thus acts as a positive control for an HTS in which various compounds are screened for their ability to displace a labeled ANT ligand.

As an example of the automated label detection systems used in the HTS assays of the Example, when the detectably labeled ANT ligand of the assay is ^{125}I -ATR, an automatic gamma counter is used. Alternatively, ^{125}I -ATR can be used in scintillation proximity assays (SPA). For example, a GST-huANT fusion protein is contacted with ScintiStrip 96-well plates coated with glutathione (EG&G Wallac). The polystyrene of these plates contains a scintillating agent that emits beta radiation when excited by a gamma emitter in close proximity thereto. The beta radiation is then detected by any appropriate automatic beta counter. When fluorescent ANT ligands are used in the HTS assay, an automatic fluorescence counter is used and may be, e.g., a FLUOROCOUNT™ Counter (Packard Instrument Company, Meriden, CT).

SEQUENCE LISTING

(inc. wildtype & PCR-amplified ANT cDNAs w/ restriction sites)

1 = huANT1 cDNA (inc. polypeptide sequence; Figure 1)

25 2 = huANT2 cDNA (inc. polypeptide sequence; Figure 1)

3 = huANT3 cDNA (inc. polypeptide sequence; Figure 1)

4 = PCR primer

TTATATCTCGAGTATGGGTGATCACGCTTGGAGCTTCCTAAAG

5 = PCR primer

30 TATATAGGTACCTTAGACATATTTTTTGATCTCATCATAACAAC

6 = PCR primer

TTATATCTCGAGTATGACAGATGCCGCTGTGTCCTTCGCCAAG

7= PCR primer

TATATAGGTACCTTATGTGTA CTTCTTGATTTCATCATAACAAG

8 = PCR primer

TTATATCTCGAGTATGACGGAACAGGCCATCTCCTTCGCCAAA

9= PCR primer

TATATAGGTACCTTAGAGTCACCTTCTTGAGCTCGTCGTACAGG

5 10 = sequencing primer

TATGCCATAGCATTTTTATCC

11 = sequencing primer

CGCCAAAACAGCCAAGCT

12 = mutagenic oligonucleotide primer

10 GGAGATGGCCTGTTCCGTCATCTTATCGTCATCGTCGTACAGATC

13 = mutagenic oligonucleotide primer

GATCTGTACGACGATGACGATAAGATGACGGAACAGGCCATCTCC

14 = PCR primer

CCCGGGGAATTCTGATGACGGAACAGGCCATCTCC

15 15 = PCR primer

CCCGGGCTCGAGTTAGAGTCACCTTCTTGAGCTC

16 = PCR primer

TTATAGGATCCATGACGGAACAGGCCATCTCCTTCGCCAAA

17 = PCR primer

20 TTAAAGAATTCTTAGATCACCTTCTTGAGCTCGTCGTACAG

18 = sequencing primer

AAATGATAACCATCTCGC

19 = sequencing primer

ACTTCAAGGAGAATTTCC

25 20 = sequencing primer

ACTTCGCCTTCACGGATA

21 = sequencing primer

TACGGCCAAGGGCATTCT

22 = sequencing primer

30 TGAAGCGGAAGTTCCTAT

23 = sequencing primer

ATGCCGGTTCCCGTACGA

24 = mutagenic oligonucleotide primer

GGCCTGTTCCGTCATCTTATCGTCATCGTCG

25 = mutagenic oligonucleotide primer

5 CGACGATGACGATAAGATGACGGAACAGGCC

26 = PCR primer

TTAAAGAATTCATGACGGAACAGGCCATCTCCTTCGCCAAA

27 = PCR primer

TTATAGGATCCTTAGATCACCTTCTTGAGCTCGTCGTACAG

10 28 = PCR primer

TTAATGGGTACCATGACGGAACAGGCCATCTCCTTCGCCAAA

29 = PCR primer

TTATACTCGAGTTAGATCACCTTCTTGAGCTCGTCGTACAGG

30 = synthetic polypeptide

15 Cys-Trp-Arg-Lys-Ile-Phe-Arg-Asp-Glu-Gly-Gly-Lys-Ala-Phe-Phe

31 = huANT1 amino acid sequence (Figure 1)

32 = huANT2 amino acid sequence (Figure 2)

33 = huANT3 amino acid sequence (Figure 3)

CLAIMS

We claim:

1. A recombinant expression construct comprising at least one regulated promoter operably linked to a first nucleic acid encoding an adenine nucleotide translocator polypeptide.
2. The expression construct of claim 1 further comprising at least one additional nucleic acid sequence that regulates transcription.
3. The expression construct of claim 2 wherein the additional nucleic acid sequence that regulates transcription encodes a repressor of said regulated promoter.
4. The expression construct of claim 1 wherein the adenine nucleotide translocator polypeptide comprises a human adenine nucleotide translocator polypeptide.
5. The expression construct of claim 4 wherein the human adenine nucleotide translocator polypeptide is ANT1.
6. The expression construct of claim 4 wherein the human adenine nucleotide translocator polypeptide is ANT2.
7. The expression construct of claim 4 wherein the human adenine nucleotide translocator polypeptide is ANT3.
8. An expression construct according to claim 1 wherein the adenine nucleotide translocator polypeptide is expressed as a fusion protein with a polypeptide product of a second nucleic acid sequence.
9. The expression construct of claim 8 wherein the polypeptide product of said second nucleic acid sequence is an enzyme.

10. The expression construct of claim 8 wherein said fusion protein localizes to membranes.

11. The expression construct of claim 10 wherein said membranes are mitochondrial membranes.

12. An expression construct according to claim 1 wherein the adenine nucleotide translocator polypeptide is expressed as a fusion protein with at least one product of a second nucleic acid sequence encoding a polypeptide cleavable by a protease, said adenine nucleotide translocator polypeptide being separable from the fusion protein by cleavage with the protease.

13. A host cell comprising a recombinant expression construct according to claim 1.

14. A host cell according to claim 13 wherein the host cell is a prokaryotic cell.

15. A host cell according to claim 13 wherein the host cell is a eukaryotic cell.

16. The host cell of claim 15 wherein the eukaryotic cell is selected from the group consisting of a yeast cell, an insect cell and a mammalian cell.

17. The host cell of claim 16 wherein the insect cell is an Sf9 cell.

18. A host cell according to claim 13 that lacks at least one isoform of an endogenous adenine nucleotide translocator.

19. A host cell according to claim 13 in which expression of at least one gene encoding an endogenous adenine nucleotide translocator isoform is substantially impaired.

20. A recombinant expression construct comprising at least one promoter operably linked to a nucleic acid molecule comprising a first nucleic acid sequence and a second nucleic acid sequence, said first nucleic acid sequence encoding an animal adenine nucleotide translocator polypeptide wherein the adenine nucleotide translocator polypeptide is expressed as a fusion protein with a polypeptide product of said second nucleic acid sequence.

21. The expression construct of claim 20 wherein the polypeptide product of said second nucleic acid sequence is an enzyme.

22. The expression construct of claim 20 wherein said fusion protein localizes to membranes.

23. The expression construct of claim 22 wherein said membranes are mitochondrial membranes.

24. The expression construct of claim 20 further comprising at least one additional nucleic acid sequence that regulates transcription.

25. The expression construct of claim 24 wherein the additional nucleic acid sequence that regulates transcription encodes a repressor of said promoter.

26. The expression construct of claim 20 wherein the adenine nucleotide translocator polypeptide comprises a human adenine nucleotide translocator polypeptide.

27. The expression construct of claim 26 wherein the human adenine nucleotide translocator polypeptide is ANT1.

28. The expression construct of claim 26 wherein the human adenine nucleotide translocator polypeptide is ANT2.

29. The expression construct of claim 26 wherein the human adenine nucleotide translocator polypeptide is ANT3.

30. An expression construct according to claim 20 wherein the adenine nucleotide translocator polypeptide is expressed as a fusion protein with at least one product of a second nucleic acid sequence encoding a polypeptide cleavable by a protease, said adenine nucleotide translocator polypeptide being separable from the fusion protein by cleavage with the protease.

31. A host cell comprising a recombinant expression construct according to claim 20.

32. A host cell according to claim 31 wherein the host cell is a prokaryotic cell.

33. A host cell according to claim 31 wherein the host cell is a eukaryotic cell.

34. The host cell of claim 33 wherein the eukaryotic cell is selected from the group consisting of a yeast cell, an insect cell and a mammalian cell.

35. The host cell of claim 34 wherein the insect cell is an Sf9 cell.

36. A host cell according to claim 20 that lacks at least one isoform of an endogenous adenine nucleotide translocator.

37. A host cell according to claim 20 in which expression of at least one gene encoding an endogenous adenine nucleotide translocator isoform is substantially impaired.

38. A recombinant expression construct according to either claim 1 or claim 20 wherein the expression construct is a recombinant viral expression construct.

39. A method of producing a recombinant adenine nucleotide translocator polypeptide, comprising:

culturing a host cell comprising a recombinant expression construct comprising at least one regulated promoter operably linked to a first nucleic acid encoding an adenine nucleotide translocator polypeptide.

40. A method of producing a recombinant adenine nucleotide translocator polypeptide, comprising:

culturing a host cell comprising a recombinant expression construct comprising at least one promoter operably linked to a nucleic acid molecule comprising a first nucleic acid sequence and a second nucleic acid sequence, said first nucleic acid sequence encoding an animal adenine nucleotide translocator polypeptide wherein the adenine nucleotide translocator polypeptide is expressed as a fusion protein with a polypeptide product of said second nucleic acid sequence.

41. A method of producing a recombinant adenine nucleotide translocator polypeptide, comprising:

culturing a host cell infected with the recombinant viral expression construct of claim 38.

42. An ANT polypeptide produced by the method of any one of claims 39-41.

43. An isolated human adenine nucleotide translocator polypeptide.

44. The isolated polypeptide of claim 43 wherein the human adenine nucleotide translocator polypeptide is recombinant ANT1 or a variant or fragment thereof.

45. The isolated polypeptide of claim 43 wherein the human adenine nucleotide translocator polypeptide is recombinant ANT2 or a variant or fragment thereof.

46. The isolated polypeptide of claim 43 wherein the human adenine nucleotide translocator polypeptide is recombinant ANT3 or a variant or fragment thereof.

47. An isolated human adenine nucleotide translocator fusion protein comprising an adenine translocator polypeptide fused to at least one additional polypeptide sequence.

48. The fusion protein of claim 47 wherein said one additional polypeptide sequence is an enzyme sequence or a variant or fragment thereof.

49. The fusion protein of claim 47 wherein said fusion protein localizes to membranes.

50. The fusion protein of claim 49 wherein said membranes are mitochondrial membranes.

51. An isolated human adenine nucleotide translocator fusion protein comprising an adenine translocator polypeptide fused to at least one additional polypeptide sequence cleavable by a protease, said adenine nucleotide translocator polypeptide being separable from the fusion protein by cleavage with the protease.

52. An isolated adenine nucleotide translocator fusion protein comprising a first polypeptide that is an animal adenine translocator polypeptide fused to at least one additional polypeptide sequence.

53. The fusion protein of claim 52 wherein said one additional polypeptide sequence is an enzyme sequence or a variant or fragment thereof.

54. A fusion protein according to claim 52 that localizes to membranes.

55. A fusion protein according to claim 54 wherein said membranes are mitochondrial membranes.

56. An isolated recombinant animal adenine nucleotide translocator fusion protein comprising an adenine translocator polypeptide fused to at least one additional polypeptide sequence cleavable by a protease, said adenine nucleotide translocator polypeptide being separable from the fusion protein by cleavage with the protease.

57. The fusion protein of either claim 47 or claim 52 wherein the additional polypeptide sequence is a polypeptide having affinity for a ligand.

58. A method for determining the presence of an ANT polypeptide in a biological sample comprising:

contacting a biological sample suspected of containing an ANT polypeptide with an ANT ligand under conditions and for a time sufficient to allow binding of the ANT ligand to an ANT polypeptide; and

detecting the binding of the ANT ligand to an ANT polypeptide, and therefrom determining the presence of an ANT polypeptide in said biological sample.

59. The method of claim 58 wherein the adenine nucleotide translocator polypeptide comprises a human adenine nucleotide translocator polypeptide.

60. The method of claim 59 wherein the human adenine nucleotide translocator polypeptide is ANT1.

61. The method of claim 59 wherein the human adenine nucleotide translocator polypeptide is ANT2.

62. The method of claim 59 wherein the human adenine nucleotide translocator polypeptide is ANT3.

63. The method of claim 58 wherein the ANT ligand comprises atractyloside substituted at 6' hydroxyl to form an atractyloside derivative.

64. The method of claim 63 wherein the atractyloside is detectably substituted at the 6' hydroxyl to form a detectable atractyloside derivative.

65. The method of claim 64 wherein the detectable atractyloside derivative comprises a radiolabeled substituent.

66. The method of claim 65 wherein the radiolabeled substituent is selected from the group consisting of ^{125}I , ^{131}I , ^3H , ^{14}C and ^{35}S .

67. The method of claim 64 wherein the detectable atractyloside derivative comprises a fluorescent substituent.

68. The method of claim 67 wherein the ANT ligand further comprises a Eu²⁺ atom complexed to the atractyloside derivative.

69. The method of claim 64 wherein the detectable atractyloside derivative comprises covalently bound biotin.

70. The method of claim 63 wherein the atractyloside molecule is substituted at 6' hydroxyl with an amine or an amine containing functionality to form an amine modified atractyloside derivative.

71. The method of any one of claims 63-70 wherein the atractyloside molecule is a carboxyatractyloside molecule that is substituted at 6' hydroxyl to form an atractyloside derivative that is a carboxyatractyloside derivative.

72. A method for isolating ANT from a biological sample, comprising:
contacting a biological sample suspected of containing an ANT polypeptide with an ANT ligand under conditions and for a time sufficient to allow binding of the ANT ligand to an ANT polypeptide; and
recovering the ANT polypeptide, and thereby isolating ANT from a biological sample.

73. The method of claim 72 wherein the ANT ligand is covalently bound to a solid phase.

74. The method of claim 72 wherein the ANT ligand is non-covalently bound to a solid phase.

75. A method for identifying an agent that binds to an ANT polypeptide, comprising:

contacting a candidate agent with a host cell expressing at least one recombinant ANT polypeptide under conditions and for a time sufficient to permit binding of the agent to said recombinant ANT polypeptide; and

detecting binding of said agent to the recombinant ANT.

76. The method of claim 75 wherein the host cell is a prokaryotic cell.

77. The method of claim 76 wherein the prokaryotic cell is an *E. coli* cell.

78. The method of claim 75 wherein the host cell is a eukaryotic cell.

79. The method of claim 78 wherein the eukaryotic cell is selected from the group consisting of a yeast cell, an insect cell and a mammalian cell.

80. The method of claim 79 wherein the insect cell is an Sf9 cell.

81. The method of any one of claims 75-80 wherein the host cell lacks at least one isoform of an endogenous adenine nucleotide translocator.

82. The method of any one of claims 75-80 wherein host cell expression of at least one gene encoding an endogenous adenine nucleotide translocator isoform is substantially impaired.

83. A method for identifying an agent that binds to an ANT polypeptide, comprising:

contacting a candidate agent with a biological sample containing at least one recombinant ANT polypeptide under conditions and for a time sufficient to permit binding of the agent to said ANT polypeptide; and

detecting binding of said agent to the recombinant ANT polypeptide.

84. A method for identifying an agent that interacts with an ANT polypeptide comprising:

contacting a biological sample containing recombinant ANT with a detectable ANT ligand in the presence of a candidate agent; and

comparing binding of the detectable ANT ligand to recombinant ANT in the absence of said agent to binding of the detectable ANT ligand to recombinant ANT in the presence of said agent, and therefrom identifying an agent that interacts with an ANT polypeptide.

85. An ANT ligand comprising atractyloside substituted at the 6' hydroxyl to form an atractyloside derivative.

86. The ANT ligand of claim 85 wherein the atractyloside is detectably substituted at the 6' hydroxyl to form a detectable atractyloside derivative.

87. The ANT ligand of claim 86 wherein the detectable atractyloside derivative comprises a radiolabeled substituent.

88. The ANT ligand of claim 87 wherein the radiolabeled substituent is selected from the group consisting of ^{125}I , ^{131}I , ^3H , ^{14}C and ^{35}S .

89. The ANT ligand of claim 86 wherein the detectable atractyloside derivative comprises a fluorescent substituent.

90. The ANT ligand of claim 89 further comprising a Eu^{2+} atom complexed to the atractyloside derivative.

91. The ANT ligand of claim 86 wherein the detectable atractyloside derivative comprises covalently bound biotin.

92. The ANT ligand of claim 85 wherein the atractyloside molecule is substituted at 6' hydroxyl with an amine or an amine containing functionality to form an amine modified atractyloside derivative.

93. An ANT ligand according to any one of claims 85-92 wherein the atractyloside molecule is a carboxyatractyloside molecule that is substituted at 6' hydroxyl to form an atractyloside derivative that is a carboxyatractyloside derivative.

94. An assay plate for high throughput screening of candidate agents that bind to at least one ANT polypeptide, comprising:

an assay plate having a plurality of wells, each of said wells further comprising at least one immobilized recombinant ANT polypeptide or a variant or fragment thereof.

95. A method of targeting a polypeptide of interest to a mitochondrial membrane, comprising:

expressing a recombinant expression construct encoding a fusion protein in a host cell, said construct comprising at least one regulated promoter operably linked to a nucleic acid molecule comprising a first nucleic acid sequence and a second nucleic acid sequence, said first nucleic acid sequence encoding an adenine nucleotide translocator

polypeptide that is expressed as a fusion protein with a polypeptide product of said second nucleic acid sequence, wherein said second nucleic acid sequence encodes the polypeptide of interest.

96. A method of targeting a polypeptide of interest to a mitochondrial membrane, comprising:

expressing a recombinant expression construct encoding a fusion protein in a host cell, said construct comprising at least one promoter operably linked to a nucleic acid molecule comprising a first nucleic acid sequence and a second nucleic acid sequence, said first nucleic acid sequence encoding an animal adenine nucleotide translocator polypeptide that is expressed as a fusion protein with a polypeptide product of said second nucleic acid sequence, wherein said second nucleic acid sequence encodes the polypeptide of interest.

97. A pharmaceutical composition comprising an ANT ligand of any one of claims 85 or 86.

98. A pharmaceutical composition comprising an agent that binds to an ANT polypeptide identified according to claim 75.

99. A pharmaceutical composition comprising an agent that binds to an ANT polypeptide identified according to claim 83.

100. A pharmaceutical composition comprising an agent that interacts with an ANT polypeptide identified according to claim 84.

101. A method of treatment comprising administering to a subject the pharmaceutical composition of any one of claims 97-100.

PRODUCTION OF ADENINE NUCLEOTIDE TRANSLOCATOR (ANT),
NOVEL ANT LIGANDS AND SCREENING ASSAYS THEREFOR

ABSTRACT OF THE DISCLOSURE

Compositions and methods are provided for producing adenine nucleotide translocator (ANT) polypeptides and fusion proteins, including the production and use of recombinant expression constructs having a regulated promoter. ANT ligands and compositions and methods for identifying ANT ligands, agents that bind ANT and agents that interact with ANT are also disclosed.

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hANT1p	MGDFAWSFTKDFLAGAVAAAMSKTAVAPIERVKLLLOVQHASKQISAEKQ	50
hANT2p	MTDAAVSFAKDFLAGGVAAAI SKTAVAPIERVKLLLOVQHASKQITADKQ	50
hANT3p	MTEQATISFAKDFLAGGIAAAI SKTAVAPIERVKLLLOVQHASKQITADKQ	50
hANT1p	YKGIIDCVVRIPKEQGFLSFWRGNLANVIRYFPTQALNFAFKDKYKQIFL	100
hANT2p	YKGIIDCVVRIPKEQGVLSFWRGNLANVIRYFPTQALNFAFKDKYKQIFL	100
hANT3p	YKGIIDCVVRIPKEQGVLSFWRGNLANVIRYFPTQALNFAFKDKYKQIFL	100
hANT1p	GGVDRHKQFWRYFAGNLASGGAAGATSLCFVYPLDFARTRLAADVGRR-A	149
hANT2p	GGVDKRTQFWLYFAGNLASGGAAGATSLCFVYPLDFARTRLAADVGKAGA	150
hANT3p	GGVDKHTQFWRYFAGNLASGGAAGATSLCFVYPLDFARTRLAADVGKSGI	150
hANT1p	QREFHGLGDCIITKIIFKSDGLRGLYQGFNVSVQGIITIRAAAYFGVYDTAKG	199
hANT2p	EREFRGLGDCIGKIIFKSDGIRGLYQGFNVSVQGIITIRAAAYFGIYDTAKG	200
hANT3p	EREFRGLGDCIMKIIFKSDGIRGLYQGFNVSVQGIITIRAAAYFGVYDTAKG	200
hANT1p	MLPDPKNVHIFVSWMIAQSVTAVAGLISYPFDTVRRRMMMQSGRKGADIM	249
hANT2p	MLPDPKNTHIVISWMIAQTVTAVAGLISYPFDTVRRRMMMQSGRKGIDIM	250
hANT3p	MLPDPKNTHIVVSWMIAQTVTAVAGVVSYPFDTVRRRMMMQSGRKGADIM	250
hANT1p	YTGTVDCWRKIAKDEGAKAFFKGAWSNVLRCMGGAFLVLVLYDEIKKYV.	298
hANT2p	YTGTILDCWRKIARDEGGKAFFKGAWSNVLRCMGGAFLVLVLYDEIKKYT.	299
hANT3p	YTGTVDCWRKIIFRDEGGKAFFKGAWSNVLRCMGGAFLVLVLYDEIKKVI.	299

Figure 2

66264-10035T00

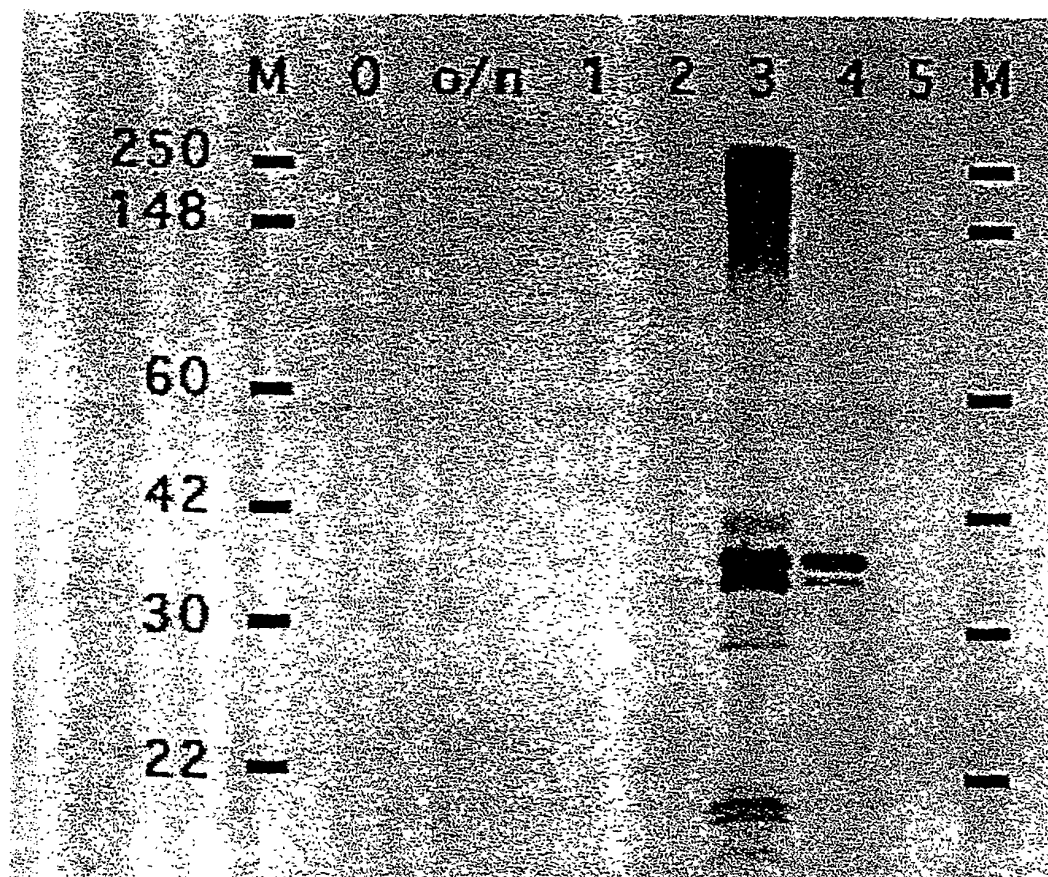


Figure 3

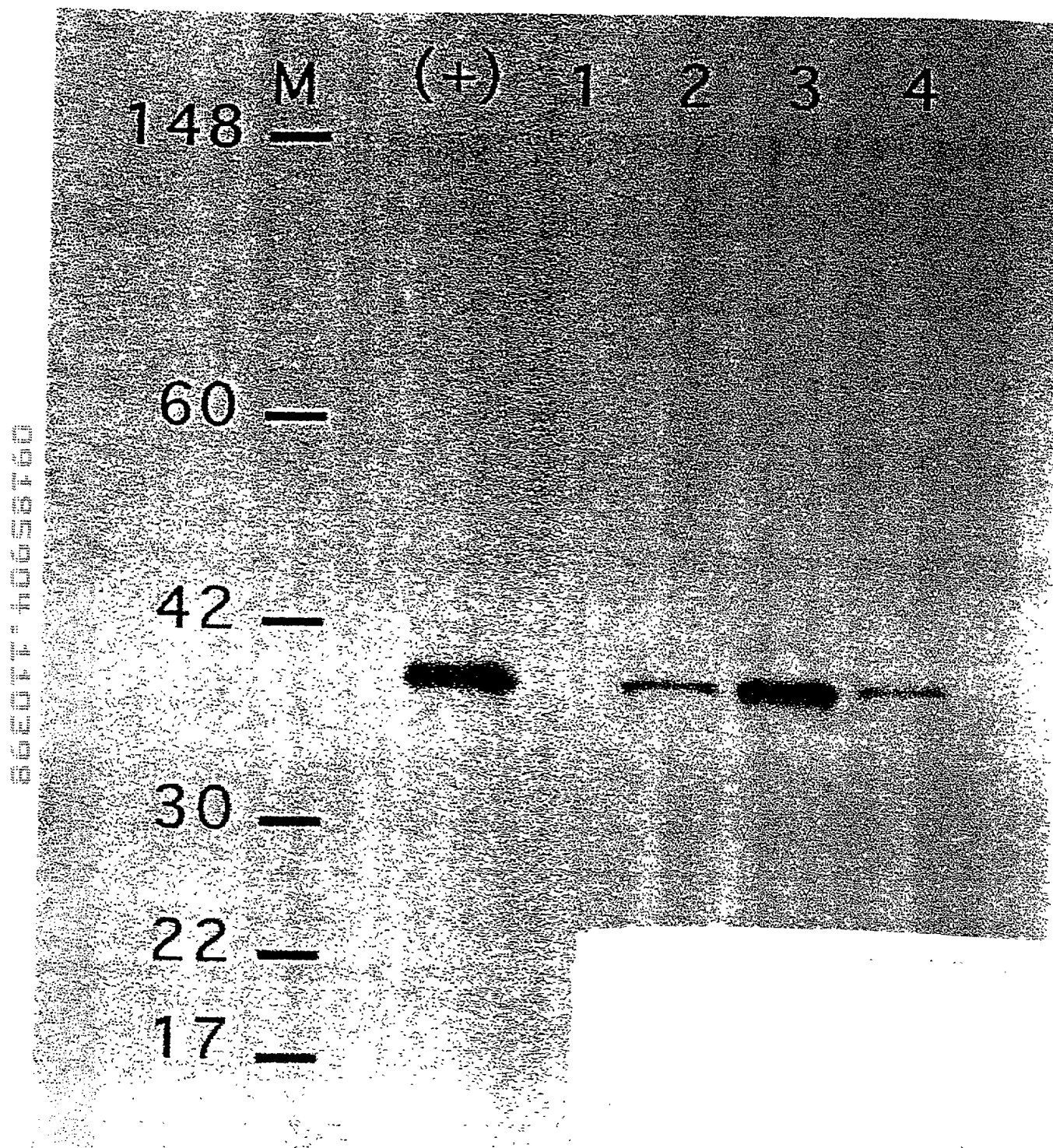


Figure 4

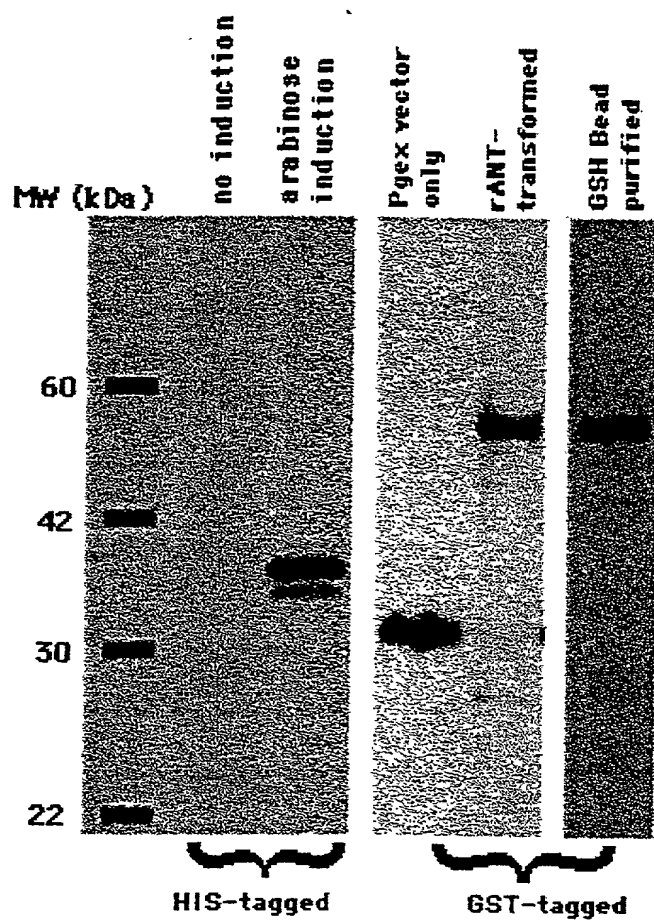


Figure 5

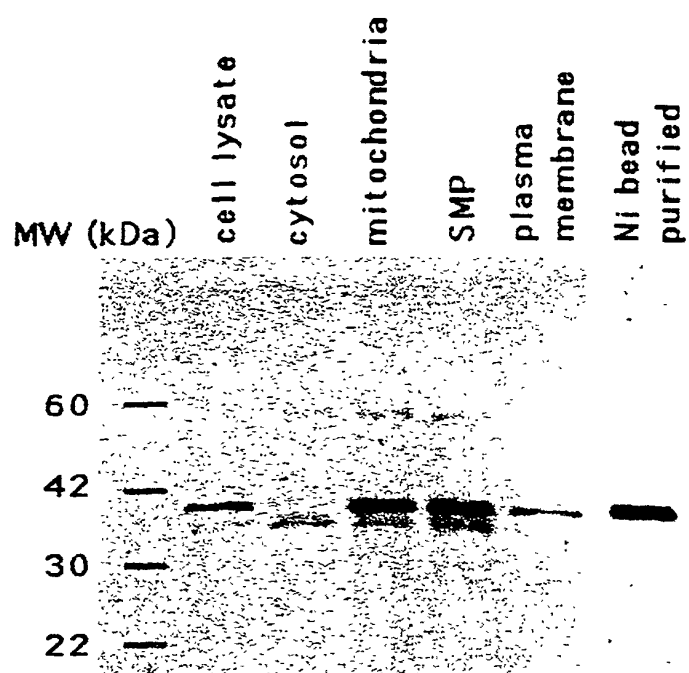


Figure 6

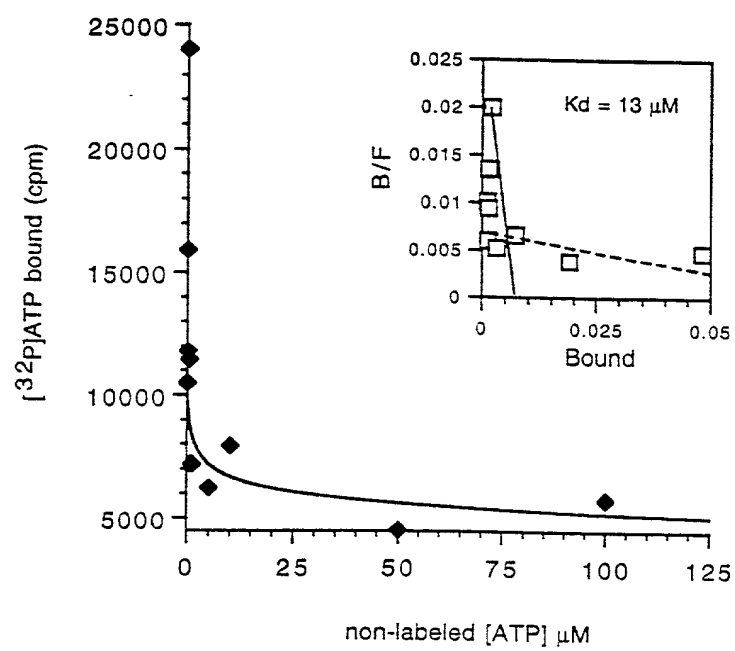


Figure 7

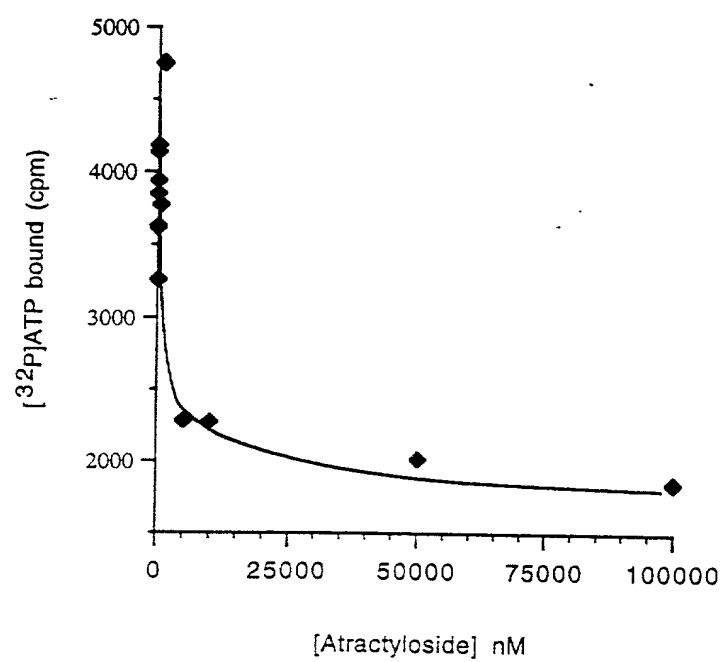


Figure 8

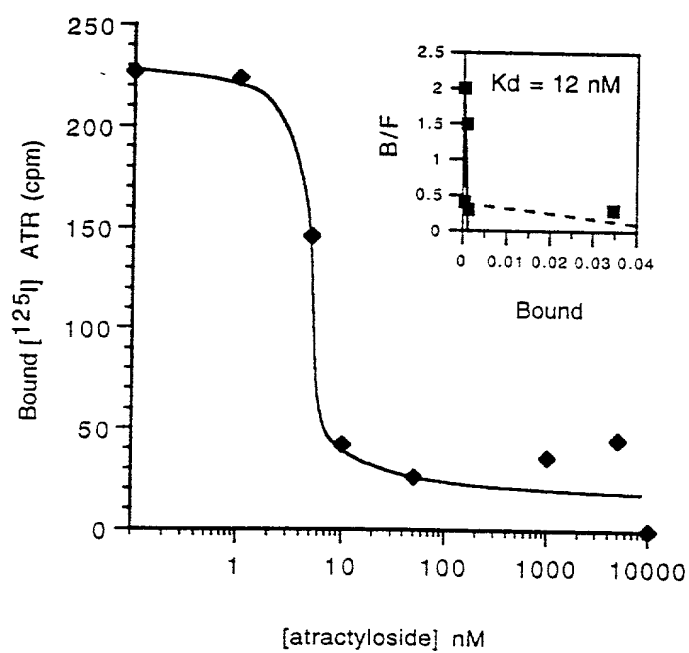


Figure 9




Figure 10